Exhibit 1

# GENERATION OF TRANSGENIC DAIRY CATTLE USING *'IN VITRO'* EMBRYO PRODUCTION

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**We have combined gene transfer, by microinjection, with *'in vitro'* embryo production technology, enabling us to carry out non-surgical transfer, to recipient cows, of microinjected embryos that have been cultured from immature oocytes. Using this approach, we have established 21 pregnancies from which 19 calves were born. Southern blot analysis proved that in two cases the microinjected DNA had been integrated in the host genome.**

Heterologous protein production in the mammary gland of dairy animals may become an important alternative to cell-culture based expression systems. The feasibility of this technology in the mouse system has been demonstrated in several reports in which high level, tissue-specific expression of foreign proteins in milk was obtained[1–5]. Several groups have reported limited success of germline transformation by applying essentially the same approach to other species, in particular animals with a relatively short generation time such as rabbits, sheep and pigs[6–11]. In these experiments, the supply of fertilized oocytes was, like that in the murine system, based on superovulation followed by *'in vivo'* fertilization. After surgical removal of the zygotes followed by microinjection, embryos were allowed to develop in the oviduct of recipients into which they were placed via another surgical procedure.

Dairy cows would seem to be the optimal species for production of very large quantities of heterologous protein in the mammary gland since they can produce over 10,000 liters of milk per year that contains 35 grams of protein per liter[12]. However, the generation interval of cattle is about 2 years and cows normally produce only one offspring per gestation. In addition, the logistics of supplying the large numbers of bovine zygotes that are required for the production of transgenic animals from live animals using conventional procedures is cumbersome. Moreover, superovulation and artificial insemination followed by flushing of oviducts of donor cows and oviductal transfer to recipients is very costly because of the two surgery steps involved.

Here we report the generation of transgenic dairy cattle based on a novel approach in which gene transfer is combined with an *'in vitro'* embryo production procedure, thus enabling non-surgical transfer of microinjected embryos that have developed from immature oocytes. Using this approach we demonstrate, for 2 cases, the successful incorporation of microinjected DNA in the genome. The integrated DNA construction contains signals for directing expression of the human iron binding protein, lactoferrin, to the mammary gland.

## RESULTS

**Oocyte maturation and fertilization.** Bovine oocytes were collected by aspiration of follicles present on ovaries obtained from local slaughterhouses. For this study a total of about 2500 oocytes were used. On average we performed two aspiration sessions per week. The yield of aspirated oocytes was highly variable from day to day, with a mean daily number of about 150. Maturation and fertilization were analyzed by cytological analysis. Maturation was defined as the breakdown of the nuclear membrane, the appearance of the first polar body and a metaphase plate. For fertilization, frozen semen from three different bulls was used, each with excellent characteristics with respect to genetic background, field perfor-

**TABLE 1** Efficiencies of the steps involved in the process from immature bovine oocytes to transgenic calves.

| Step | Total No. | Percent* |
|---|---|---|
| oocytes | 2470 | — |
| matured | 2297 | 93 |
| fertilized | 1358 | 61 |
| injected | 1154 | 85 |
| survival | 981 | 85 |
| cleavage | 687 | 70 |
| transferred | 129‡ | 19 |
| pregnant | 21 | 21 |
| integration | 2 | 10 |

*Percentages indicate the proportion of embryos or cells that successfully complete each step.
‡Sixty-nine transfers of single blastocytes resulting in 7 pregnancies; 30 transfers of twinned embryos, resulting in 14 pregnancies.

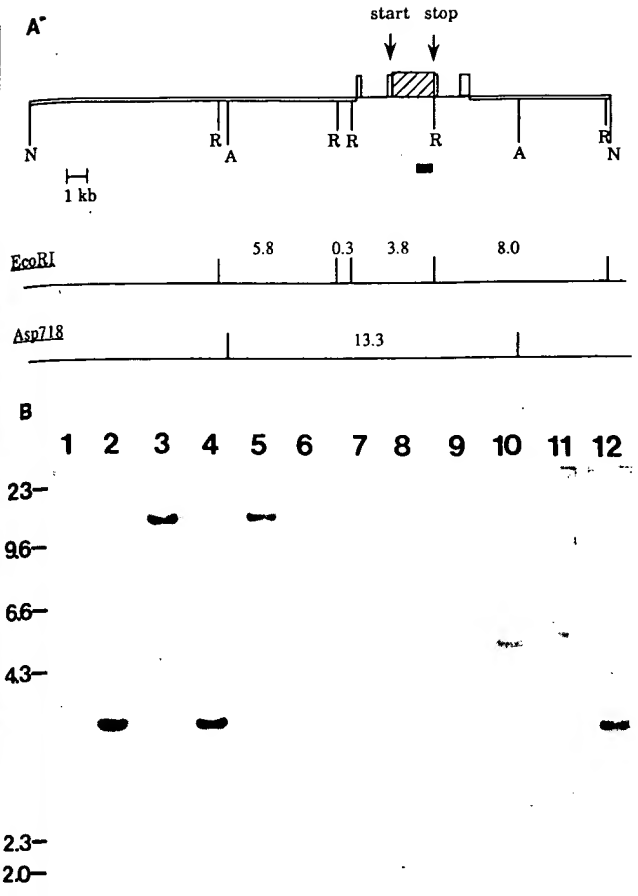


**FIGURE 1** (A) Structure of the bovine casein-hLF transgene. The coding sequence of the hLF cDNA is depicted by a hatched box. The position of the translational start and stop codon is indicated. The 5′ and 3′ untranslated regions are encoded by αS1-casein exons (open boxes). Intervening sequences interrupting these exons are represented by a single line. The expression unit is surrounded by flanking sequences derived from the bovine αS1-casein gene (indicated by a double line). Positions of restriction enzyme sites are indicated by the following symbols: R, EcoRI; A, Asp718; N, NotI; The NotI sites are not present at the indicated positions in the bovine αS1-casein gene itself, but were introduced by synthetic linkers. The black bar represents the position of the probe used to detect the presence of the transgene. Sizes of the fragments (in kbp) obtained after digestion with EcoRI or Asp718 are shown at the bottom. (B) Southern blot analysis of DNA extracted from various tissues. Ten μg of DNA was loaded per lane. Fragment size markers in kbp. (HindIII digest of lamda DNA) are indicated on the left. Lane 1, EcoRI digested human DNA (isolated from blood), lane 2, Eco RI digested DNA from calf #4 isolated from blood; lane 3, Asp718 digested DNA from calf #4 isolated from blood; lane 4, EcoRI digested placental DNA from calf #4; lane 5, Asp718 digested placental DNA from calf #4; lane 6, EcoRI digested DNA from calf #15 isolated from blood; lane 7, Asp718 digested DNA from calf #15 isolated from blood; lane 8, EcoRI digested DNA from calf #15 isolated from ear tissue; lane 9, Asp718 digested DNA from calf #15 isolated from ear tissue; lane 10, EcoRI digested placental DNA from calf #15; lane 11, Asp718 digested placental DNA from calf #15; lane 12, EcoRI digested DNA isolated from the tail of a transgenic mouse harboring the same construct.

mance and ease of calving. For each batch of semen the *'in vitro'* fertilization conditions (heparin concentration and sperm number) were optimized to obtain normal fertilization rates ranging from 50 to 70% as determined by the presence of two pronuclei and a sperm tail. We used either one of two techniques for selection of motile sperm: the swim-up technique[13] and centrifugation through a Percoll gradient (J. Parrish, personal communication). No significant differences in fertilization rates between these methods were recorded. The efficiencies of these and the following steps are shown in Table 1.

**Pronuclear injection.** In order to visualize pronuclei, fertilized oocytes were centrifuged at 14,500× g for 8 minutes[14]. The time window in which pronuclei could be visualized appeared to be smaller than the period in which murine pronuclei are visible. Cytologically, i.e. after fixation and staining with aceto-orcein, pronuclei are clearly detectable from 16 hours up to 23 hours after fertilization, whereas using interference contrast optics pronuclei can be visualized after centrifugation between 18 hours and 23 hours after fertilization. The number of oocytes in which pronuclei could be visualized during this period was about 10% smaller than the number that was expected based on the cytological data. Possibly, some nuclei are hidden behind the yolk cap that is formed after centrifugation. Pronuclear injection was performed essentially as described[15]. About 10% of the zygotes collapsed after microinjection and were discarded.

**Embryo development.** After microinjection, the embryos were transferred to microdrops of medium conditioned by bovine oviductal epithelial cells as described[16]. Embryo development was evaluated 9 days after the start of maturation. The development rate varied by about 5% depending on the sperm donor used. In addition, microinjected embryos developed, in general, slower than control embryos that had been centrifuged but not microinjected. The formation of the blastocyst cavity was delayed by approximately 1 day. Also, fewer microinjected embryos developed to the morula/blastocyst stage than control embryos (19% vs. 20–25%, Table 1).

**Embryo transfer.** The synchronization schedule was set up so that recipients started estrous on the same day at which oocytes were aspirated from slaughterhouse ovaries (i.e. start of maturation is day 1). Recipients received 9-days old embryos, at which time they have developed to the compact morula or early blastocyst stage. These embryos are one day ahead in development compared to the stage of the estrous cycle of the recipients. In case of two microinjection sessions on subsequent days, one group of recipients was used that were in synchrony with the first batch of oocytes collected. Transfers of embryos that developed from oocytes aspirated on the day of the start of estrous gave better results than embryos from oocytes obtained one day later. Due to the somewhat delayed development of microinjected embryos, there appeared to be a better synchrony between the recipients and the first group of embryos. Recipients received two embryos when the quality grade (according to Linder and Wright[17]) was fair to poor and only one single embryo when the quality grade was excellent to good. Each pregnant recipient that received 2 embryos carried only one fetus to term. The overall pregnancy rate was 21%, which is significantly less than the rates reported by others with non-microinjected embryos which had developed *'in vivo'*[18,19]. In the experiments reported here, no transfers with non-injected embryos were performed.

A total of 21 pregnancies were established (confirmed by rectal palpation 45–60 days after transfer). During pregnancy 2 fetuses were lost. One recipient aborted spontaneously for unknown reasons at 7.5 months of gestation. The second fetus, collected at slaughter of the recipient at 3 weeks after the calculated day of parturition, was a full grown dead calf having an abnormal embryonic development called 'schistosoma reflexum'. In both cases no intact DNA could be isolated for analysis. Nineteen calves were born after normal pregnancies. One of these calves died during parturition, and a second, 24 hours after birth, because of pneumonia following accidental

inhalation of milk. A third calf, born after a pregnancy of 10 months and with a body weight of 70kg was euthanized at an age of 3 weeks. Pathological analysis indicated that the animal was suffering from sepsis due to chronic omphalephlebitis. Tissues that could be analyzed from the three dead calves contained no integrated human lactoferrin (hLF) sequences. Therefore, the cause of their death is unlikely to be related to transgene integration. The remaining 16 calves are in excellent health.

**Structure of the transgene.** The fragment used for injection was designed to express hLF in the mammary gland of a lactating cow. It consists of the coding region of the hLF cDNA[20] fused to the bovine $\alpha S_1$-casein signal sequence and flanked by the 5′ and 3′ untranslated regions of the bovine $\alpha S_1$-casein gene. Both untranslated regions are interrupted by an intervening sequence. These introns were included since several groups have shown that the presence of intervening sequences can dramatically increase expression of cDNA-based constructs both in tissue culture and in transgenic animals[21,22].

Expression of the cDNA is controlled by regulatory elements within 15 kbp of 5′-flanking and 6 kbp of 3′-flanking sequences from the $\alpha S_1$-casein gene. These sequences have been shown to contain elements that are responsible for tissue-specific expression of heterologous genes in transgenic mice[5] (and our own unpublished observations). A schematic drawing of the casein-hLF transgene is shown in Figure 1.

**DNA analysis.** DNA was isolated from placenta, blood and ear tissue from all calves. Southern blot analysis using hLF cDNA as a probe indicated that in tissues of two calves (#4 and #15) transgene sequences had been integrated into the host genome. Calf #15 (a female) was mosaic for integration of the transgene: placental tissue was positive, whereas in blood and ear tissue no hLF sequences could be detected. The copy number in the placenta was 1–2. The restriction enzyme map of the transgene was different from that expected based on the map of the casein-hLF plasmid (Fig. 1) and based on the pattern obtained in many individual transgenic mice (data not shown). Apparently, a rearrangement had occurred involving a deletion of part of the DNA construct. It is not clear whether this rearrangement event is related to the fact that the transgene could not be detected in all tissues. In mice it has been shown that over 30% of all transgenic animals born are mosaic[23].

Calf #4 (a male) showed, in all three tissues, the same hybridization pattern that was identical to the expected one. Restriction digestions with different enzymes indicated that head-to-tail concatemers of intact copies had integrated and there was no indication of rearrangements. Copy numbers were estimated by comparing the intensities of the transgenic band with bands resulting from hybridization of the hLF probe to human DNA (Fig. 1). In calf #4 between 5 and 10 copies of the transgene had integrated in all three tissues examined.

## DISCUSSION

This work proves the technical feasibility of transgenesis in the bovine system: in 2 out of 19 calves born from microinjected zygotes, the introduced DNA was integrated into the host genome. In parallel experiments, the transgenesis rate in mice that received the same casein-hLF construct was about 10% (data not shown). Thus, based on the limited number of animals born, we tentatively conclude that the transgenesis rates in cattle and mice are, in our hands, the same.

Most attempts to produce transgenic cattle have relied partly or totally on *in vivo* procedures[24,25]. Fertilized oocytes were retrieved from superovulated and artificially inseminated cows. Microinjected zygotes were transferred by surgery either directly into the oviduct of recipient cows or into temporary hosts like sheep or rabbits. Obviously, the *'in vivo'* production of zygotes is much more labor intensive than the *'in vitro'* production. Also a larger number of additional animals and veterinary surgeons are involved. Another major disadvantage of the *'in vivo'* procedure is that the stage of pronuclear development at which the zygotes are isolated and processed varies considerably[26], and therefore the most suitable time for microinjections cannot be determined. In addition, up to 50% of the embryos transferred into ligated oviducts in living animals cannot be recovered[27]. Finally, the development of the embryos cannot be followed in the oviduct of temporary hosts, and therefore the stage at which the embryos are recovered is unpredictable. Some of the disadvantages of the *'in vivo'* procedure may be compensated to some extent by the fact that embryos produced by this method have, in general, a better developmental potential[28].

All the disadvantages described above are circumvented when using the *'in vitro'* procedures described here. A large number of aspirated oocytes are matured and fertilized simultaneously and their pronuclei can be injected within a short time. Development of the individual embryos can be monitored closely, which makes it possible to transfer embryos at very specific stages of development. The *'in vitro'* culture of embryos up to the morula/blastocyst stage also allows for additional manipulations such as biopsy of blastomers for transgenesis detection, sexing and cloning. It is expected that the incorporation of these, in part established, technologies in our *'in vitro'* program will further reduce the number of recipients needed to generate (larger numbers of) transgenic cattle.

The procedures described in this paper make use of oocytes obtained from ovaries of slaughtered cows. As a consequence, the genetic background of these animals is unknown. Recent developments in the efficient retrieval of oocytes from live animals using echoscopy[29,30] make it possible to generate transgenic calves that have a defined genetic background both from the maternal and the paternal lineage.

## EXPERIMENTAL PROTOCOL

**Oocyte source.** Ovaries were collected at a local abattoir and transported to the laboratory in a insulated container at 30–32°C. Oocytes, together with follicular fluid, were aspirated from 2–8 mm diameter follicles and pooled into 50 ml conical tubes. Cumulus-oocyte complexes (COC) were allowed to settle into a pellet, after which the supernatant was discarded and the pellet washed in 50 ml TL-Hepes[30]. COC, containing several intact, unexpanded cumulus cell layers, were selected and isolated under a dissecting microscope at 15× magnification, washed four times in 10 ml TL-Hepes, once in 2–3 ml TCM199+10% fetal calf serum (M199)[30] and then transferred to 100 µl droplets of M199 medium under paraffin oil (20 COC/droplet). COC were incubated for 23 h in a humidified atmosphere of 5% $CO_2$ in air at 39°C.

***'In vitro'* fertilization.** Oocytes were fertilized with frozen thawed-sperm obtained from three different bulls in artificial insemination service. Sperm capacitation was facilitated with heparin[13]. Since sperm from individual bulls respond differently to specific fertilization conditions, semen from each lot was tested in advance to determine optimal heparin and sperm concentration required to maximize normal fertilization frequency and to minimize polyspermy. Fertilization conditions for a given bull were selected after screening at heparin concentrations of 0.0, 1.0 and 10.0mg heparin/ml, and at 1.0, 2.0 and 4.0 × $10^6$ motile sperm/ml. Since the proportion of sperm that survives freezing and thawing varies from bull to bull (approximately 30–60% for the bulls used here) sperm preparations were enriched for live, motile sperm by a "swim-up" procedure[13]; alternatively, sperm were centrifuged through a percoll gradient (J. Parrish, personal

communication). After isolation of the motile portion, sperm were counted on a hemocytometer, diluted to an appropriate concentration to yield a 25-fold concentrated stock. The fertilization medium consisted of TALP medium[31] supplemented with 2.0–10.0 mg/ml heparin (from porcine intestinal mucosa, 177 IU/mg; Sigma)[13] and if the cumulus was removed prior to fertilization, 1mM hypotaurine, 10mM penicillamine, 20mM epinephrine and 2mM sodium metabisulfite. Matured COC were selected on the basis of expanded cumulus masses for fertilization, washed once in 10 ml fertilization medium, and either added directly to fertilization droplets, or first stripped of their cumulus investment by gentle pipetting through a small-bore, fire-polished pipet and then added to the droplets. Finally, sperm cells were added to a final concentration of $1 \times 10^6$–$2.0 \times 10^6$/ml. After 16–24 h, presumptive zygotes were removed from fertilization droplets. At this point, 20–30 zygotes for each experiment were fixed in 3:1 ethanol:acetic acid for 24 h, stained with 1% aceto-orcein (in 40% acetic acid), and examined to determine fertilization frequency (percentage of sample with 2 pronuclei and a sperm tail). The remaining oocytes were then prepared for microinjection.

**Microinjection.** The 26 kbp casein-hLF fragment used for microinjection was released by NotI digestion and purified by agarose gel electrophoresis and electroelution. The final DNA concentration was adjusted to 2.5 μg/ml. Batches of 50 cumulus-intact fertilized oocytes were stripped either as described above or by vortexing 2 minutes in 2ml Tl-hepes medium in a 10ml conical tube. In order to visualize the pronuclei, cumulus free oocytes were centrifuged in 1ml TL-hepes medium 8 minutes at 14,500× g in an Eppendorf centrifuge[14]. Microinjection was performed essentially as described[15].

**Embryo culture.** Embryos were cultured from the zygote to the compact morula or blastocyst stage in oviductal-tissue conditioned medium[16]. Oviducts were obtained at slaughter and transported at ambient temperature. Luminal tissue from 2–4 oviducts (1–2 cows) was harvested by gently scraping intact oviducts on the outside with a glass slide. The extruded material was washed 5 times in 10 ml TALP-Hepes and diluted in M199 to a tissue:media ratio of 1:50. Media were conditioned in 50 ml "T" flasks containing five ml of oviduct tissue suspension. Conditioned medium was prepared 48 h later from the supernatant after centrifuging tissue suspensions at 13000× g for 10 min., divided into 1.0 ml aliquots and stored at −20°C until used. Conditioned media frequently contained a proteinaceous precipitate after thawing, which was removed by centrifugation. Droplets were covered with paraffin oil and were incubated for 2 h to permit pH to equilibrate prior to adding zygotes. Zygotes were placed in culture droplets within 2 h after microinjection. Initial cleavage (>2 cells) was assessed 42 h after adding sperm. Media were not changed during the course of incubations. Criteria for normal development consisted of attainment of the compact morula or blastocyst stage.

**Embryo transfer.** Estrous in recipient cattle was synchronized with a 9-day Norgestamet (Intervet, Boxmeer, The Netherlands) treatment (administered in an ear implant according to the manufacturer), and a 500 μg dose of cloprostanol given on day 7 of the Norgestamet treatment. Estrous occurred within 2–3 days after implant removal. Embryos were transferred non-surgically to recipient heifers 5–7 days after estrous (1–2 embryos/uterine horn). Pregnancy was determined by rectal palpation at 45 to 60 days of gestation.

**DNA analysis.** DNA extraction, Southern blot analysis and hybridization were performed according to standard procedures[32]. The probe used in the Southern blotting experiment was a 758 bp EcoRV-EcoRI fragment covering the 3′ part of the hLF cDNA[20].


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Exhibit 2

# PRODUCTION OF TRANSGENIC CATTLE BY PRONUCLEAR INJECTION

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Previously, this laboratory reported success in producing transgenic bovine fetuses (Biery et al., 1988, Theriogenology 29:224). Since that time, an effort was made to produce live, transgenic cattle. Data for two ova sources and four gene constructs are included. The first ova source was from excised oviducts of cattle stimulated with FSH. These ova were collected at 36 hrs post onset of estrus (24 hr post initial breeding). The second source of ova was IVM-IVF. IVM-IVF ova were subjected to pronuclear injection beginning at 18 hrs post IVF. Structural genes used were human estrogen receptor (HER) and insulin-like growth factor-I (IGF-I). Two promoters from the chicken α-skeletal actin gene were utilized, consisting of 202 bP of the promoter (202-ASK) or the promoter and first intron of the actin gene (733-ASK). Approximately 2 Kb of the mouse mammary tumor virus 3′ long terminal repeat promoter (MMTV) was also used. Injected ova were cultured in an oviductal cell co-culture. At the end of culture, morulae and blastocysts were transferred non-surgically to synchronous recipients. After calving, blood and tissue samples were analyzed for the presence of a transgene by Southern blot analysis.

| Construct | Ova Source | No. Ova Collected | No. Ova Injected (%) | No. Ova Developing (%) | No. Preg./ No. Abort | No. Live Calves | No. Transgenic |
|---|---|---|---|---|---|---|---|
| ASKHER | In-vivo | 4150 | 1878 (45) | 266 (14) | 82/12 | 53 | 1 |
| 202-ASK-IGF-I | In-vivo | 2668 | 1346 (50) | 205 (15) | 62/21 | 35 | 1 |
| 202-ASK-IGF-I | IVM-IVF | 5142 | 2559 (50) | 178[a] (7) | 44/8 | 32 | 2[b] |
| MMTV-IGF-I | In-vivo | 538 | 246 (46) | 38 (15) | 14/5 | 9 | 1 |
| MMTV-IGF-I | IVM-IVF | 798 | 667 (84) | 63 (9) | 15/2 | 11 | 1 |
| 733-ASK-IGF-I | In-vivo | 295 | 136 (46) | 15 (11) | 6/4 | 2 | 0 |
| 733-ASK-IGF-I | IVM-IVF | 5775 | 4374 (76) | 293 (7) | 92/34 | 51 | 1[b] |

[a]40 Not transferred.
[b]These calves were stillborn or died within one day of birth.

Ova from IVM-IVF procedures were more synchronous as evidenced by a high proportion of injected ova. In-vivo ova had higher development and pregnancy rates. No gross abnormalities were observed in any calves. Seven animals tested positive for the transgene. The efficiency of producing these transgenic animals (#Transgenic/Ova Injected) ranged from 0.02 to 0.18%. Two bulls lived to sexual maturity. Semen was collected and frozen from these bulls and used to produce embryos through IVM-IVF and co-culture. These embryos were analyzed by PCR for presence of the transgene. Results for a bull with ASKHER showed 34 of 74 embryos to possess the transgene, and for the bull with 202-ASK-IGF-I 64 of 114 embryos possessed the transgene. These data show that transgenic cattle can be produced by pronuclear injection which are capable of transmitting the transgene to progeny.

Exhibit 3

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# Transgenic Cattle Resulting from Biopsied Embryos: Expression of c-ski in a Transgenic Calf

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## ABSTRACT

Producing transgenic cattle by microinjection of DNA into pronuclei has been inefficient and costly, in large part because of the cost of maintaining numerous nontransgenic pregnancies to term. We designed a system for early identification of transgenic embryos in which biopsies of embryos were assayed by polymerase chain reaction for presence of the transgene before embryo transfer. A total of 2555 embryos were microinjected with one of two DNA constructs. Of the 533 embryos biopsied, 112 were judged to be potentially transgenic and were transferred nonsurgically to recipients, resulting in production of 29 putative transgenic fetuses. One fetus and one calf (7% of offspring) were subsequently shown to be definitively transgenic. The calf was transgenic for a chicken c-ski cDNA, and several months after birth developed dramatic muscular hypertrophy followed by muscle degeneration. This phenotype was associated with expression of high levels of mRNA from the transgene.

## INTRODUCTION

The success of modern animal agriculture is largely a result of many centuries of selection for desirable genotypes. With the advent of transgenic technology, it became appealing to consider accelerating the pace of such genetic progress, including modifying the genome of animals in ways that probably cannot be done by classical means. Cattle represent a very substantial fraction of world agricultural output and are therefore an attractive target for genetic manipulation. However, progress in producing transgenic cattle is constrained by a number of factors, including long generation interval, low fecundity, and the expense of such work. Nevertheless, a small number of transgenic cattle have been produced in recent years [1–3]. To date, none of these transgenic cattle has been reported to express RNA or protein from the transgene.

A large part of the expense in conducting transgenic research with livestock is in maintaining to term the recipient animals that harbor nontransgenic fetuses. This is a particularly serious problem with cattle because efficiency, in terms of the fraction of pregnancies that are transgenic, has been very low, and the cost of maintenance is high. Considerable savings could be realized if embryos could be reliably screened for transgenic status before transfer. Here we report production of a transgenic calf and a transgenic fetus derived from embryos that were biopsied prior to embryo transfer to determine transgenic status.

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## MATERIALS AND METHODS

### *Embryo Manipulation*

Embryos for microinjection were obtained by either surgical recovery from superovulated cows or by in vitro maturation and in vitro fertilization of oocytes aspirated from ovarian follicles of slaughtered cows. In the former case, cows were superovulated according to a protocol similar to that previously described [4], consisting of intramuscular injections at half-day intervals of 6, 6, 4, 4, 2, 2, 2, and 2 mg of FSH (FSH-P; Schering-Plough, Kenilworth, NJ) beginning near midcycle. Prostaglandin $F_{2\alpha}$ was administered to induce luteolysis, and 100 μg of GnRH was administered intramuscularly at the onset of estrus. Animals were artificially inseminated one-half day and one day after the onset of estrus. Donor cows were anesthetized 48–54 h after the onset of estrus and subjected to midline laparotomy, and embryos were recovered by retrograde flushing of the oviducts with Dulbecco's PBS supplemented with 0.3 mM pyruvate and 0.2% BSA. Embryos derived from in vitro fertilization were generated essentially as described [5] and were microinjected 18–26 h after mixing of oocytes and sperm.

Microinjection was conducted similarly to what has previously been described for pig and sheep embryos [6, 7]. One-cell embryos were centrifuged for 3 min at 12 500 × *g* to polarize cytoplasmic lipid droplets, and then microinjected into one pronucleus with a solution of the DNA fragment (2 ng/μl in 10 mM Tris [pH 7.4], 0.2 mM EDTA). Two-cell embryos were treated similarly and injected into one or both nuclei. The majority of embryos were then processed through the following system. After microinjection, embryos were placed in 50-μl drops of oviduct epithelial cell-conditioned medium [8] under paraffin oil and cultured overnight. The next day, cleaved embryos were transferred to the ligated oviducts of estrous rabbits. Embryos

were recovered from rabbits 6–7 days later (equivalent to 9–10 days from the onset of donor estrus) and evaluated for stage of development. Morulae and blastocysts were microsurgically biopsied by techniques similar to those previously described for producing identical twin cattle [9]. Briefly, embryos were placed in a 10-cm plastic Petri dish containing protein-free PBS, resulting in sticking of zonae pellucidae to the plastic surface. With the aid of a micromanipulator, a small fragment of razor blade attached to a glass pipette was used to cut a biopsy from the side of the embryo, avoiding the inner cell mass. Typically, the biopsy was estimated visually to consist of 10–30 of the embryo's trophoblast cells. While the biopsied embryos remained in culture, the biopsies were analyzed for the presence of the transgene by polymerase chain reaction (PCR). Embryos classified as strong or weak by PCR were transferred nonsurgically to the uterus of recipient cows that generally were one day behind the donor cow in estrous cycle synchrony. In a majority of cases, single embryos were transferred, but often one or two poor-quality embryos were transferred with a good embryo to the same recipient. Pregnancy was diagnosed by ultrasonography 30–40 days after transfer. Most of the recipients that became pregnant after transfer of putative transgenic embryos were subjected to allantocentesis 70–90 days after transfer. For this procedure, a flank laparotomy was performed under local anesthesia, and 20–30 ml of allantoic fluid was aspirated through a 20-gauge needle attached to a piece of polyethylene tubing; the first 5–10 ml of fluid was discarded to minimize contamination with maternal cells. Fetal cells were collected from the fluid by centrifugation, counted, and assayed by PCR. Fetuses from other recipients were either recovered surgically between 70 and 130 days of gestation or allowed to go to term.

### *ransgenes*

Embryos were injected with one of two DNA sequences. The first was a construct designed to express the bovine leukemia virus (BLV) envelope protein under control of an immunoglobulin regulatory element (IgSVBLVenv), and was constructed with use of standard techniques by ligating sequences encoding the BLV gp51 gene into an expression vector consisting of the SV40 early promoter and mouse immunoglobulin heavy chain enhancer (Schnieke, unpublished). Expression of gp51 from this construct in cultured cells was found to be very poor after its use for microinjection into bovine embryos was initiated. The second construct, MSVski/Δ29, consisted of a truncated form of chicken c-ski under transcriptional control of a murine sarcoma virus regulatory region [10, 11]; this construct was provided by Drs. Pramod Sutrave and Stephen Hughes (NCI, Frederick, MD). For microinjection, both constructs were released from plasmid sequences, isolated from agarose gels, and purified by adsorption and elution from glass particles.

### *PCR and DNA Hybridizations*

Embryo biopsies (approximately 10–30 cells) and cells collected by allantocentesis (approximately 500 cells) were placed in 0.5-ml polypropylene tubes in less than 5 μl of PBS. PCR was initiated by adding a mixture of buffer and primers and heating the samples to 95°C for 7 min in a thermocycler (Perkin-Elmer-Cetus, Irvine, CA). The samples were then cooled to 80°C and held at that temperature while nucleotide triphosphates and *Taq* polymerase was added. Samples were then subjected to 35 cycles of amplification (94°C/1 min, 55°C/1.5 min, 72°C/2.5 min) with a final 10-min extension at 72°C. Final concentrations of reactants in a 100-μl volume were as follows: single-strength buffer, 1.5 mM MgCl, 200 μM for each nucleotide, 200 nM for each primer, and 2.5 U *Taq* polymerase. Positive (plasmid) and negative (no DNA) control samples were included in each assay. The primers used for detection of MSVski were 5′-AAGGAATTCTCTAGCACGATTGAG (forward) and 5′ CTCAGTAGAAGCTGGAGATTG (reverse). At completion of amplification, 25–30 μl of each product was electrophoresed in 2% agarose containing ethidium bromide, and fluorescent bands of the appropriate size were scored qualitatively as clearly positive or questionable (faint).

Extraction of genomic DNA and Southern blot analyses were conducted according to standard procedures [12]. Ten-microgram samples of DNA were restricted overnight, electrophoresed in 0.8% agarose, and transferred to nylon membranes (Gene Screen Plus, Dupont, Wilmington, DE) by capillary blotting. These samples were hybridized overnight to chicken c-ski cDNA probes labeled with $^{32}P$ by the random priming method. After a final wash in single-strength saline sodium citrate/0.1% SDS at 55°C, blots were subjected to autoradiography for 1–3 days.

## RESULTS

### *Production and Screening of Transgenic Embryos*

A total of 1573 surgically collected and 982 in vitro-fertilized embryos were microinjected with DNA. Of these, 875 embryos were injected with IgSVBLVenv, a construct designed to express the bovine leukemia virus envelope glycoprotein, and 1680 were injected with MSVski, a vector for expression of a truncated form of chicken c-ski. Of 2046 embryos transferred to rabbits, 1535 (75%) were recovered, and 533 of those had developed into blastocysts of quality sufficient to biopsy (approximately 32% and 15% of the transferred embryos derived from oocytes fertilized in vivo and in vitro, respectively). Transfer of 112 PCR-positive or PCR-questionable embryos into 88 recipients (66, 20, and 2 transfers of one, two or three embryos, respectively) resulted in establishment of 27 pregnancies with 29 fetuses. Twelve of these fetuses were derived from embryos injected with IgSVBLVenv and 17 from embryos injected with MSVski DNA. To investigate the possibility of false negative diagnoses, 84

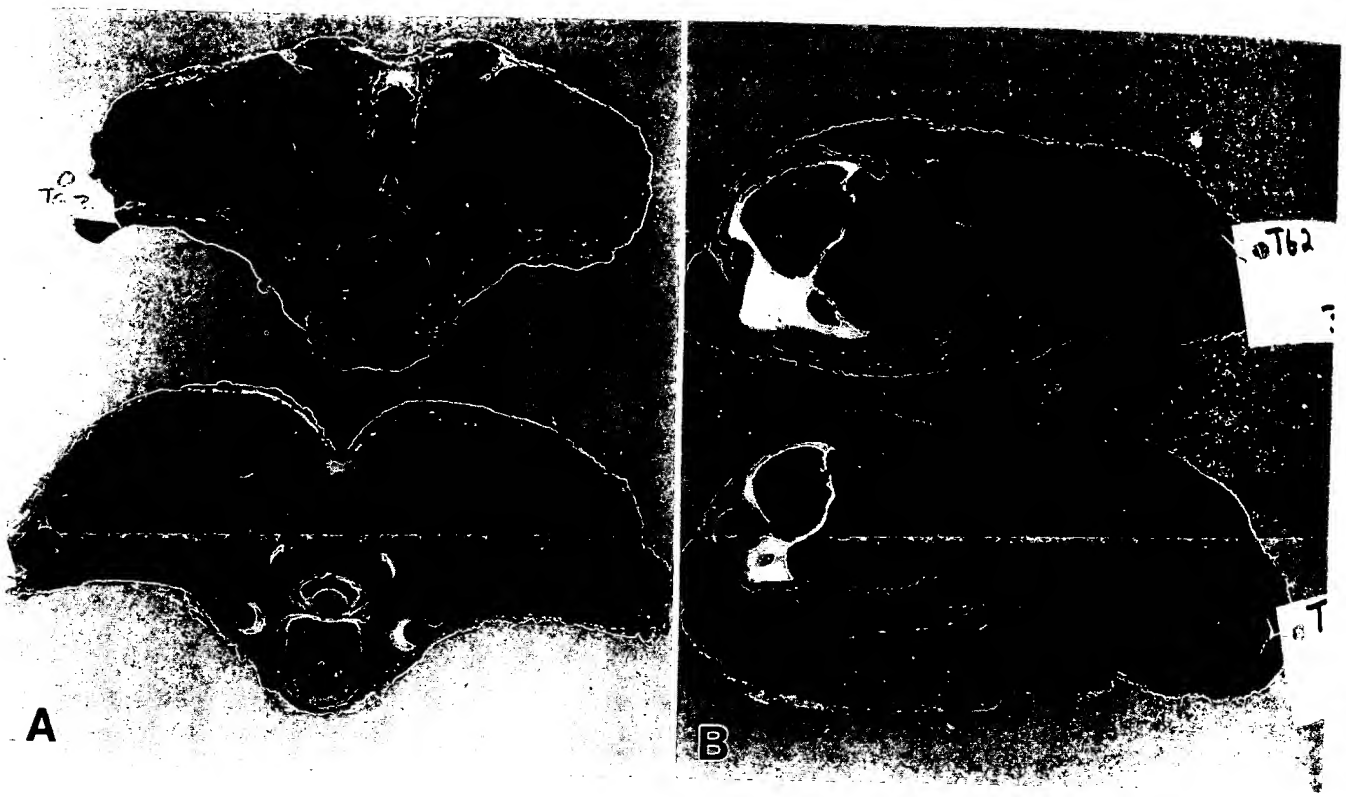


FIG. 1. Characteristics of the carcasses from ski-transgenic calf (bottom of pairs) and his nontransgenic twin sister (top of pairs; sections are at slightly different planes in the carcass). A) Section between 12th and 13th ribs; major muscle is longissimus dorsi. B) Section through shoulder at level of scapulo-humeral joint.

biopsied embryos that had been microinjected with MSVski DNA were subjected to PCR analysis after their biopsy was classified as negative. Two of these embryos were classified as putative transgenics on the repeat assay.

Classifying blastocysts as transgenic or not by PCR was of concern because a positive result might only reflect detection of a small amount of injected DNA that had persisted in the embryo but was not integrated. We therefore chose to evaluate each of the putative transgenic pregnancies again at 60–80 days of gestation. Each of the 12 fetuses derived from microinjection of IgSVBLVenv DNA was recovered surgically, and DNA extracted from multiple tissues (liver, skin, and cotyledon) was evaluated by Southern hybridization. One of these fetuses was shown to be transgenic, with the same pattern of hybridizing restriction fragments in all three tissues. The transgene was not detected in the remaining 11 fetuses of this group.

To assess transgenic status in the fetuses from embryos injected with MSVski, fetal cells collected by allantocentesis were again analyzed by PCR. Cells from one of the fetuses contained DNA that was specifically amplified using c-ski primers. This pregnancy consisted of male and female twins. All 17 MSVski pregnancies were allowed to proceed to term. At birth, a skin or tail biopsy was obtained from each of the calves, and DNA extracted from that sample was examined by PCR with c-ski primers. None of the 15 calves diagnosed as nontransgenic at 60–80 days of gestation was transgenic at birth. However, a specific PCR amplification product was obtained with DNA of both twin calves from the pregnancy identified as transgenic by allantocentesis. Southern hybridization with radiolabeled c-ski cDNA and DNA extracted from skin, and at a later age from liver, was used to show that the male calf was transgenic and that the transgene was intact. The female calf showed a small vulva and vagina, and at necropsy, marked uterine and ovarian hypoplasia, characteristics typical of a freemartin. A very weak hybridization signal was obtained with DNA extracted from her skin and a relatively strong signal from liver DNA, undoubtedly due to the hematopoietic chimerism that usually occurs in twin bovine pregnancies; this calf was not considered to be truly transgenic.

*Phenotypic Changes Associated with Expression of c-ski*

The c-ski transgenic bull calf was normal at birth except for the presence of mild micro-ophthalmia, a defect that is relatively common in cattle and was most likely not associated with his being transgenic. This calf remained phenotypically normal during the first 8 wk of life. However, over the following 2 wk, indications of muscular hypertrophy became evident, particularly affecting the loins and rear quarters. At approximately 10 wk of age, the bull began to manifest sporadic periods of weakness that, over a 2-wk pe-

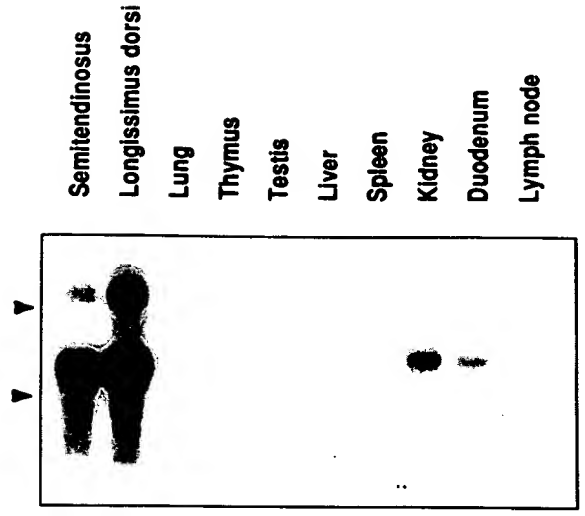


FIG. 2. Northern blot analysis of RNA extracted from tissues collected from ski-transgenic calf. Twenty micrograms of total RNA was loaded in each lane, and blots were hybridized to radiolabeled chicken c-ski cDNA. Arrows mark position of ribosomal RNA bands.

riod, progressed to an inability to stand without assistance. Serum concentrations of creatine phosphokinase during this period were significantly elevated (ranging from 808–897 IU/L in comparison to 118–171 IU/L in sera from his nontransgenic twin), suggestive of skeletal muscle degeneration or damage. Throughout this period, the bull remained alert, manifested no signs of pain, and retained a normal appetite. At 15 wk of age, it became obvious that the muscle weakness was not a transient phenomenon, and humane considerations led us to euthanize this animal and, for control purposes, his twin.

Gross evaluation of the carcass from the transgenic bull revealed substantial muscular hypertrophy, which was symmetrical and involved most of the major muscles associated with the axial and appendicular skeleton (Fig. 1). The cross-sectional area of the longissimus dorsi muscle between the 12th and 13th ribs was approximately 40% greater in the transgenic calf than in his nontransgenic twin (50 $cm^2$ vs. 36 $cm^2$).

Histopathologic examination of major muscles from the transgenic calf revealed almost all fibers to appear swollen, with a distinctly rounded rather than polygonal outline. A small number of individual fibers were clearly degenerative, with an accompanying mild accumulation of mononuclear cells at their periphery. Many other fibers appeared to be in earlier stages of degeneration, often showing a more acidophilic staining and distinct separation of myofibrils.

Expression of the transgene was assessed by Northern analysis using total RNA (Fig. 2). Messenger RNA for c-ski was present in high concentrations in all axial and appendicular skeletal muscles tested (semimembranosus, semitendinosus, longissimus dorsi, psoas major, triceps, and gluteus medius), as well as in diaphragm. Lesser, but still substantial levels were found in kidney and left ventricle, whereas small quantities were present in small intestine, liver, and spleen. Ski mRNA was not detected in cerebrum, lung, thymus, testis, or lymph node from the ski-transgenic calf, nor in major skeletal muscles (semimembranosus, semitendinosus, and longissimus dorsi) of the nontransgenic twin calf. Transgenic c-ski mRNA was predominantly of the size expected (approximately 2.9 kb), although a larger hybridizing species of unknown origin (approximately 6.5 kb) was observed in muscle samples containing high levels of c-ski mRNA.

## DISCUSSION

The study reported here suggests that it is feasible to screen embryos for presence of a transgene prior to transfer into recipients. We generated two transgenic animals from a total of 2555 embryos injected with DNA, an efficiency similar to that reported by other groups using bovine embryos [2, 3]. However, viewed from a different perspective, these two transgenic cattle were derived from 29 fetuses that were obtained from embryos biopsied as blastocysts to assess transgenic status. If the biopsy and PCR step had not been included, we probably would have transferred all 533 good-quality embryos recovered from rabbits to recipient cows (Table 1). Introduction of the embryo biopsy step into the protocol thus did not increase overall efficiency, but it significantly reduced the expense of performing this work by eliminating much of the cost associated with maintaining nontransgenic pregnancies to term.

The PCR technique used for analyzing biopsies is extremely sensitive, but incapable of differentiating integrated DNA from residual input DNA. Short-term persistence in the embryo of small quantities of the microinjected DNA was

TABLE 1. Efficiencies of key steps in producing transgenic calves.

| | N | Percent of original embryos | Percent of previous step |
|---|---|---|---|
| Embryos microinjected with DNA | 2555 | 100 | 100 |
| Embryos transferred to rabbits | 2046 | 80 | 80 |
| Embryos recovered from rabbits | 1535 | 60 | 75 |
| Embryos biopsied and subjected to PCR | 533 | 21 | 35 |
| Embryos transferred to recipient cows | 112 | 4.4 | 21 |
| Putative transgenic pregnancies | 29 | 1.1 | 26 |
| Trangenics produced | 2 | 0.1 | 7 |

probably responsible for our finding that only 2 of the 29 embryos (7%) that were PCR-positive at biopsy and developed into fetuses were truly transgenic when examined later in gestation. This supposition was supported by PCR on bovine embryos at increasing times after microinjection: 5 of 6, 5 of 6, 5 of 8, 4 of 8, and 2 of 8 embryos were PCR-positive when assayed 1, 2, 3, 5, and 7 days, respectively, after microinjection. Additional experimental support for the problem of plasmid persistence has recently been provided from other laboratories [13, 14]. Despite the problem of false positives, the system of assessing transgenic status in embryos before transfer to recipients reduced the number of recipients needed by 79 percent, as 421 of the 533 good-quality embryos recovered from rabbits were not transferred.

Another important question related to diagnosing transgenic status by embryo biopsy is how frequently the procedure fails to detect truly transgenic embryos, which are then discarded. A substantial percentage of transgenic mice have been found to be mosaics [15]. Integration of a transgene selectively or predominantly into cells that form either inner cell mass or trophoblast could result in a biopsy of trophoblast being falsely classified as negative or positive. We detected the transgene by PCR in 2 embryos from a sample of 84 in which the transgene was not detected in the original biopsy. This could have resulted either from a false negative assay from the biopsy or from transgene mosaicism. Regardless of cause, it is probable that application of the protocol described here will result in discarding an occasional embryo that is truly transgenic. Additionally, there are several ways in which this protocol could be improved, albeit at the cost of increased complexity or time. For instance, a second set of primers could be included as an internal control to co-amplify a segment of bovine genomic DNA. Similarly, Y chromosome-specific primers could be included to sex the embryo, which should allow routine transfer of more than one embryo without the risk of generating freemartins.

The transgenic calf obtained carried a truncated cDNA for the chicken c-ski gene, under regulatory control of murine sarcoma virus promoter and enhancer sequences. This was the same construct used by others to generate transgenic mice [10] and pigs [16]. As might be expected, development of muscular hypertrophy in mice transgenic for chicken c-ski varied with level of expression. The mice that developed muscular hypertrophy showed high levels of c-ski RNA in muscle, whereas those that were transgenic but without the phenotype had low levels of expression. A similar relationship was observed in the c-ski-transgenic pigs. It thus appears that a relatively high threshold of ski gene expression is necessary for development of a hypertrophic phenotype. The ski-transgenic calf reported here supports these observations in that widespread muscle hypertrophy was found in association with high levels of expression of the c-ski transgene. In this calf, as well as in some of the ski-transgenic pigs, high levels of ski gene expression we also associated with muscle dysfunction and degeneratic The pathogenesis of this phenomenon is unclear, partic larly in view of the current lack of understanding of h ski fits into normal patterns of muscle development a interacts with other transcription factors [17–19]. It is like that a window of expression exists in which muscular h pertrophy will develop without impaired function. Belo this window, muscular hypertrophy will not be observe and above it, pathologic changes will ensue. The width this window has significant implications for utility of th gene in transgenic food animals.

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# Generation of Transgenic Dairy Cattle from Transgene-Analyzed and Sexed Embryos Produced *In Vitro*

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**We have generated a transgenic calf from *in vitro* produced bovine embryos which had undergone transgene analysis and sexing prior to the embryo transfer. Bovine oocytes were isolated from slaughterhouse-derived ovaries, matured and fertilized *in vitro* and subsequently microinjected with a *dam*-methylated gene construct consisting of genomic sequences encoding human erythropoietin and governed by bovine αS1-casein regulatory sequences. After 6 to 7 days in culture, the embryos were biopsied and while the embryo remained in culture, the biopsy was subjected to transgene analysis and sexing. The transgene analysis was accomplished with a combined treatment of the embryo lysates with DpnI restriction endonuclease and Bal31 exonuclease followed by polymerase chain reaction (PCR). The transgene analysis was based on the fact that DpnI only cleaves its recognition sequence if the adenine in the sequence is methylated. Pregnancy was induced by the transfer of three viable female embryos with a distinct transgene signal to a hormonally synchronized heifer recipient. Amniotic fluid analysis performed two months after the embryo transfer confirmed the presence of the transgene. The calf born was found to be transgenic by PCR analysis from blood, ear and fetal membranes. The presence of the transgene was also confirmed by Southern blotting.**

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With the advent of transgenic technology, the production of valuable proteins of pharmaceutical or industrial interest in large farm animals has become an attractive alternative to microbial and animal cell bioreactors. During the past few years, several transgenic domestic animals with targeted transgene expression have been created. These include transgenic sheep producing high quantities of human α1-antitrypsin in the milk[1], transgenic goats expressing in their mammary gland human tissue plasminogen activator[2] and transgenic dairy cattle harboring a human lactoferrin gene governed by bovine αS1-casein regulatory sequences[3]. Similarly, a succesful generation of transgenic swine with high level expression of human protein C in their milk has recently been reported[4]. The production of substantial amounts of human hemoglobin has been accomplished with the aid of transgenic pigs, in which the human hemoglobin level in the circulation accounted for up to 9% of the total hemoglobin[5]. The generation of transgenic farm animals is, however, prohibitively expensive because of the long gestation period, small litter size and high maintenance costs of these animals. The common use of *in vivo* matured embryos likewise requires large number of donor animals and makes the embryo supply limited. In the case of dairy cattle, the latter has been overcome by using *in vitro* matured and fertilized bovine oocytes which have been subsequently cultured *in vitro* to a stage suitable for embryo transfers[3]. However, even with this practically unlimited supply of embryos, because of the very low transgenesis rate in farm animals, a large number of recipients is required for embryo transfers. This bottleneck could be simply solved by a reliable screen of the embryos for transgene integration prior to transfer. Several attempts have been made to establish such a transgene detection system for the embryos of large domestic animals[6,7]. The use of direct PCR with transgene-targeted primers for embryo selection has been shown to yield a high number of false positives[6,7]. Better selection of true transgene-positive embryos can be accomplished by the use of *dam* methylated gene constructs for micro-injections and DpnI digestion of embryo biopsies prior to the PCR analysis[8].

We now report the succesful generation of transgenic dairy cattle developed from *in vitro* cultured embryos which have been screened for transgene and sex prior to embryo transfer.

## Results

**Production and analysis of bovine embryos.** After *in vitro* maturation and fertilization (as described[9]), the bovine zygotes were microinjected with a gene construct consisting of bovine αS1-casein promoter and polyadenylation signal sequences combined with the genomic sequences of the human erythropoietin gene. Chicken lysozyme A element coupled to mouse mammary tumor virus hormone response element was cloned 5′ to the bovine αS1-casein regulatory sequences (Fig. 1). Altogether 1198 *in vitro* fertilized zygotes were produced on two consecutive days and 859 (71%) of them contained visible pronuclei and were microinjected. For the sexing and transgene analysis, 82 morulae and blastocysts were biopsied (9.5% of the injected zygotes), and 69 biopsies were analyzed. The internal control signal was visible in 57 biopsies and 7 embryos (embryos 6, 7, 10, 17, 19, 24 and 36) displayed distinct transgene-signals (12% transgenesis) (Fig. 2). Besides distinct

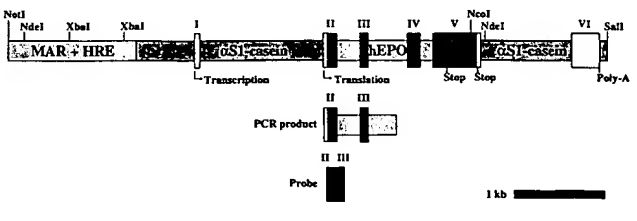


**FIGURE 1. The structure of the bovine casein–human erythropoietin (AHRECASEPO) gene construct. White boxes represent casein and black boxes erythropoietin exons. MAR, chicken lysozyme A element 1.3 kb HindIII-XbaI fragment[12]; HRE, mouse mammary tumour virus hormone response element (bases 7112-7295 from pMSG, Pharmacia, Sweden); α-S1-casein, bovine α-S1-casein regulatory sequences bases -610-1483 and 16301-17665 (ref. 13); hEPO, human erythropoietin coding sequences bases 1269-2961 (ref. 14). The AHRECASEPO specific PCR product is 819 bp in size and the probe hybridizes to 3.9 kb NcoI-XbaI and 5.1 kb NdeI fragments.**

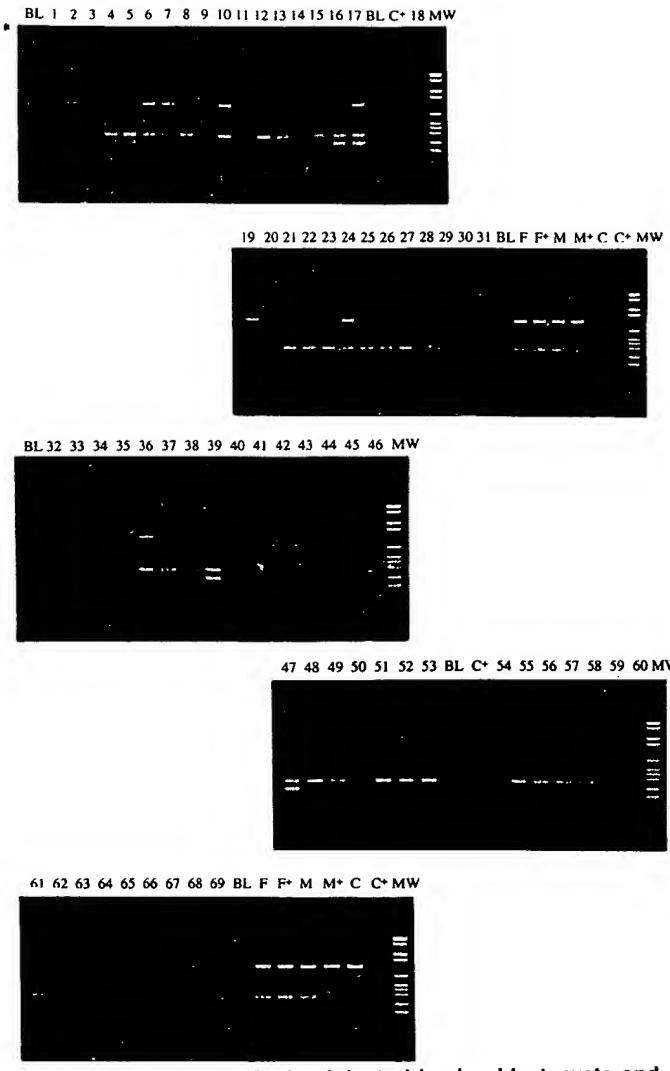


**FIGURE 2. Analysis of microinjected bovine blastocysts and compact morulas with digestion-PCR[9]. Lysed embryonic biopsies (samples 1–69) were digested with DpnI-Bal31 mixture before amplification with the primers 695 (5′-TTACCTGTCTTGTGGCTGTTGCT-3′), 220 (5′-GTGCAGTGGTGTGATCACAGCT-3′), 458 (5′-TGGACCCAGGCAAAGAGACTAG-3′), 337 (5′-CACGCTGCAATTCCAATACACAGAG-3′) and 338 (5′-CAAGCTAATCGATCCATCCTATAGTC-3′). Primers 695 and 220 amplify a 819 bp product from the transgene, primers 695 and 458 amplify a 370 bp product from bovine αS1-casein and primers 337 and 338 amplify a 287 bp product from bovine Y-chromosome. BL, lysate buffer; C, microinjection DNA (12 fg); F, female bovine DNA (70 pg) mixed with transgenic mouse DNA (140 pg); M, male bovine DNA (70 pg) mixed with transgenic mouse DNA (140 pg); MW, molecular weight markers (pBR328 BglI + pBR328 HinfI). The control samples (C, F and M) were analyzed with (+) and without DpnI-Bal31 digestion.**

transgene-signals, 5 questionable signals (Fig. 2, embryos 2, 9, 34, 41 and 57) were detected in the analysis. Thereafter, only viable embryos with a distinct transgene-signal were transferred to two hormonally synchronized heifer recipients, one receiving one male embryo and the other three female embryos.

**Generation of transgenic calf.** Pregnancy was achieved with the heifer receiving female embryos (Fig. 2, embryos 6, 7 and 36) and the presence of the transgene was confirmed in the amniotic fluid analysis (Fig. 3) after two months. All the analyzed samples from the subsequently born calf were transgene-positive by PCR (Fig. 3) and also by Southern analysis (Fig. 4). The estimated transgene copy number in the calf was one, and because the transgene locus contained only one copy, the calf was probably not mosaic. The weaker signal in Figure 4 track 3 is likely due to the lesser amount of DNA in the sample. The animal was healthy and the hematocrit was in the normal range (38%).

**Reliability of the screening assay.** Between October 1992 and November 1993 we microinjected and analyzed about 10,000 fertilized oocytes, and with transferable embryos were able to produce three calves plus one established pregnancy. The transgenesis rate among analyzed embryos varied substantially between experiments, from 2.6 to 35.7%, average being 15.4%. The first two pregnancies were produced with one viable embryo, one giving a questionable (visible, but very weak) transgene signal and the other a somewhat stronger signal by *in vitro* analysis. Subsequent analysis of the amniotic fluid samples of these fetuses showed that they were transgene-negative as were the born calves. We have since confirmed that biopsied embryos giving questionable PCR signals are, for the most part, transgene negative. The third pregnancy, resulting from the transfer of embryos with distinct transgene-signals, produced the transgenic calf.

## Discussion

The production of transgenic farm animals in general and transgenic dairy cattle in particular is extremely costly and labor-intensive, mainly due to the long gestation period, small litter size, low transgene integration rate and technical difficulties related to the embryo manipulations. Some of the difficulties, such as the limited availability of fertilized bovine oocytes, can be overcome to some extent by maturing and fertilizing slaughterhouse-derived oocytes *in vitro*, as we and some others[3] have done. However, even with the unlimited availability of bovine zygotes, compromises have to be made with regard to the low development rate of *in vitro* produced embryos. As a random transfer of embryos developed from microinjected zygotes requires a large number of recipient animals owing to the low transgene integration rate, a reliable screening of the embryos for transgene integration prior to transfer would be important. Direct PCR analysis with primers targeted to the transgene construct, however, gives unacceptable high rates of false positives[6,7]. And our own results have indicated that more than 70% of microinjected bovine blastocysts, and 30–70% of uninjected control blastocysts exposed to the microinjection DNA in the injection chamber are transgene-positive according to direct PCR analysis. Only control blastocysts which had never been exposed to exogenous DNA at any stage were consistently PCR negative (unpublished). Even the inclusion of DpnI digestion of the *dam* methylated gene construct prior to PCR analysis is not reliable enough in our experience. As utilized here, the inclusion of both DpnI and Bal31 exonuclease in the assay system appears to give the best results, providing that the digestion conditions have been properly determined for each individual gene construct. As biopsing *in vitro*-produced bovine embryos decreases embryonic viability to some extent, the low viability can be compensated by transferring multiple embryos to each recipient. In that case, however, it is essential to sex the embryos and transfer only embryos of the same sex to one recipient in order to avoid freemartinism. In practice, sexing of preimplantation bovine embryos in conjunction with the transgene analysis requires no additional efforts, provided that the primers are compatible with each other. The inclusion of αS1-casein sequences as internal controls makes the analysis more reliable as unsuccesful assays due to lost samples, biopsies of poor quality and problems in PCR are not judged as false negatives. The large variation in transgenesis rate between experiments may be attributable to the differencies in *in vitro* development of embryos and to the different persons doing microinjections. Generally, the poorer the embryonic development after microin-


**FIGURE 3. PCR analysis of amniotic fluid sample and tissues of the transgenic calf. The controls (1–3 and 5) and calf samples (4, 6–9) were amplified for 32 cycles with the primers 695, 220, 458, 337 and 338. 1, Female bovine DNA (1 ng); 2, male bovine DNA (1 ng); 3, transgenic mouse DNA (10 ng, one AHRECA-SEPO copy/cell); 4, aminotic fluid (60 µl) lysate; 5, lysate buffer; 6, blood (0.75 µl) lysate; 7, ear lysate; 8 and 9, placenta lysate; MW, molecular weight markers (pBR328 BglI + pBR328 HinfI). The transgene specific PCR product is 819 bp, the bovine α-S1-casein specific PCR product is 370 bp and the bovine Y-chromosome specific PCR product is 287 bp in size.**

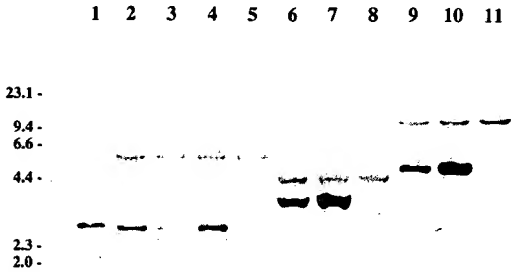


**FIGURE 4. Southern blot analysis of the transgenic calf. DNA (15 µg) were prepared from the transgenic calf's placenta (2), ear (3) and blood (4, 6 and 9) and from the blood of a non-transgenic cow (5, 8 and 11). Transgenic mouse (one AHRECA-SEPO copy/cell) DNA (1) and non-transgenic cow DNA mixed with 30 pg microinjection DNA (7 and 10) were used as gene copy number controls. After digestion with EcoRI (1–5), NcoI + XbaI (6–8) and NdeI (9–11), the samples were electrophoresed, transferred to positively charged Nylon membrane and hybridized with digoxigenin labeled single stranded PCR probe. The probe was detected using Lumigen PPD as a substrate. Transgene specific hybridization products are 2.8 kb (1–5), 3.9 kb (6–8) and 5.1 kb (9–11) in size.**

jection, the higher is the transgenesis rate.

By initially transferring gene-injected, whole embryos into 30 recipients we obtained 17 pregnancies. However, none of the resulting calves was transgenic. The good pregnancy efficiency but the lack of transgenic offspring may perhaps be explained by the fact that only the fastest developing, best quality embryos were transferred. Our later observations have indicated that the transgenesis frequency among the fastest developing embryos is less than 5% according to the screen employed here (unpublished observation). The frequency of successful pregnancies dropped significantly after we started to transfer only biopsied, transgene-positive embryos; so far 27 transfers have resulted in only 4 pregnancies. At present, the absolute reliability of our *in vitro* screen is impossible to judge. However, even with a succesful screening of preimplantation bovine embryos for transgene integration and sex, thousands of microinjections have to be carried out to obtain a single pregnancy with a transgene-positive embryo.

## Experimental Protocol

**Production of microinjected, transgene analyzed and sexed bovine embryos.** A detailed protocol is described elsewhere[9]. Briefly, oocytes were collected from slaughterhouse ovaries, matured and fertilized with frozen-thawed bull semen. The zygotes were vortexed, centrifuged and pronucleus-injected with the AHRECASEPO-gene construct (2µg/ml) into one of the pronuclei. The zygotes were subsequently cultured in 50-µl co-culture drops with bovine oviductal epithelial cells[10]. Compact morulae and blastocysts were biopsied 7 to 8 days after fertilization with a microblade under steromicroscope by hand. The biopsies, representing about one third of the embryo, were collected into lysate buffer tubes, whereas the remaining embryos were cultured until embryo transfers. Lysed biopsies were digested with DpnI (30 mU) -Bal31 (100 µU) mixture before PCR amplification with transgene, αS1-casein and Y-chromosome specific primers for 35 cycles. The results, the presence of the transgene and the embryonic sex, were visualized after agarose gel electrophoresis of the PCR products.

**Embryo transfers and amniocentesis.** After overnight culture viable transgene positive embryos were transferred non-surgically to the uterine horn ipsilateral to the corpus luteum of the recipients, which had been hormonally synchronized with two cloprostenol injections (Estrumat®, 0.5 mg/recipient) twelve days apart. The amniotic samples were aseptically aspirated from the amniotic cavity through a flank incision under local anaesthesia at two months of pregnancy.

**DNA preparation and analysis.** PCR analyses from aminotic fluid and calf samples were carried out using lysates made in PCR-buffer containing proteinase K (200 µg/ml). Ear and placental tissue was used directly, but blood samples (50 µl) were diluted 1:10 with 10 mM-Tris 1 mM-EDTA pH 8.0 buffer and centrifuged, whereas aminotic fluid samples (2 ml) were centrifuged, before suspending into lysate buffer. The samples were amplified for 32 cycles in 50 µl volume directly after proteinase K digestion (2 h +60°C). For Southern blot analysis, genomic DNA was prepared with proteinase K digestion and phenol/chlorophorm extraction from placenta, ear and blood. After restriction enzyme digestions, the samples were electrophoresed on 0.9% agarose gels and transferred to positively charged Nylon membranes (Boehringer Mannheim) using capillary transfer. A single stranded digoxigenin-labeled probe (Fig. 1) was prepared with PCR, hybridizations and subsequent chemiluminescence detections using Lumigen PPD (Boehringer Mannheim) as a substrate were done as recently described[11].

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Exhibit 5

# Transgenic Livestock as Bioreactors: Stable Expression of Human Alpha-1-Antitrypsin by a Flock of Sheep

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**We have previously described the generation of transgenic sheep expressing human alpha 1-antitrypsin ($h\alpha_1AT$) in their milk. Here, we report the fidelity of transgene transmission and expression by these animals and their progeny. Transgene transmission has been demonstrated in four of six ovine lines studied. Three of these four lines have exhibited stable transmission of the transgene, whereas the fourth has produced some offspring with reduced copy numbers. Sequential lactations of founder animals has yielded very similar levels of $h\alpha_1AT$ protein in milk. Moreover, in one line, derived from a founder male, a flock of seven G1 ewes have yielded comparable levels of $h\alpha_1AT$ protein in first and second lactation milk. Two G2 ewes of this line have also produced levels of human protein equivalent to their mother. Although the inheritance of the same transgene in mice was reminiscent of the situation in sheep, stable expression was observed in only one of four lines studied. The importance of these observations to the use of transgenic livestock as bioreactors for the production of human proteins is discussed.**

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The development of methods for the production of transgenic mammals has contributed to understanding the factors which control the expression of specific genes and in the identification of genes and processes which regulate cell proliferation, differentiation and interactions. The advent of this technology has also stimulated research into the use of transgenic, domestic livestock as bioreactors to produce large amounts of human proteins in various extracellular body fluids. The generation of a transgenic mammal usually involves the introduction of foreign DNA directly into the fertilized egg by microinjection[1], or the use of embryonic stem cells[2], a method so far only successful in mice. The major difference between these approaches is that the latter permits the targeting of events to specific chromosomal locations[3-5] whereas the former results in apparently random integration events. Very often, the microinjection approach results in variable expression from identical constructs in distinct lines of transgenic mice. This effect is often attributed to the so called "position effect" resulting from the lack of transcriptional control elements, incorrect chromatin structure or combinations of these and reasons unknown. The inclusion of matrix attachment elements[6] or locus control regions[7] into constructs has to some degree alleviated these problems.

Despite a great deal of effort from many groups around the world, the development of embryonic stem cells for livestock has so far been unsuccessful. Accordingly, the recent extension of this technology to the production of human proteins in transgenic livestock has so far been limited to the microinjection route. Thus far, the two most popular methods have been to direct expression to haematopoietic cells[8] or to the lactating mammary gland[9-11]. The former approach can make use of DNA sequence elements to counter position effects[8]. The latter approach cannot directly profit from this as such sequence elements have not, as yet, been identified for the milk protein genes used as vectors. This is an important distinction. Consistent levels of expression from the same construct in related animals bred for a production flock are highly desirable for many reasons, not least ease of processing. Despite claims that such expression would be the norm, this has not been clearly demonstrated for any construct to date. Moreover, stable inheritance of transgenes in livestock at a structural level has only been observed in a limited number of cases[9,12] (J. Clark, pers. comm.).

We have previously reported the successful generation of sheep transgenic for $\alpha$1-antitrypsin ($h\alpha_1AT$) expressing significant quantities of the human protein in their milk throughout their lactation period[10]. This protein is essentially similar to human $\alpha$1-antitrypsin purified from human plasma[13]. Expression of the transgene involved, AATB, was less variable in the four founder sheep described than in the nine murine lines discussed[10]. This led us to suggest that expression of this transgene in transgenic sheep would give rise to more consistent and higher levels of human protein in milk than that observed with transgenic mice. We have now followed the inheritance and expression of AATB in four murine lines and six ovine lines. Three of the murine lines inherit the transgene in a stable fashion but only one exhibits stable levels of expression from lactation to

lactation and from generation to generation. Moreover, host genetic background significantly influences expression levels. Of the four sheep lines in which we have demonstrated transmission to date, inheritance of the transgene is similar to that observed in mice, with one line appearing to be unstable. However, data from sequential lactations and those of subsequent generations support the proposal that expression in the ovine system would be more consistent. Thus, a mini-flock of seven ewes containing the same integration event all express the human protein at 13–17 g/l in their milk during their first and second lactations.

## Results

**Generation of murine lines transgenic for AATB: inheritance of transgene and expression levels.** The transgenic mouse is used as a predictive model for the production of human proteins in the milk of transgenic livestock because of the lack of suitable mammary cell lines and the relative rapidity with which one can obtain milk from G0 founder animals. To date, eleven murine lines have been generated for the AATB transgene exhibiting different copy numbers and levels of expression of the human protein in their milk. These are summarized in Table 1.

**TABLE 1. Variation in hα$_1$AT expression in founder transgenic mice.**

| Founder | Copy number | hα$_1$AT Milk Concentration (μg/ml) 1st | 2nd | 3rd |
|---|---|---|---|---|
| 56-4 | ~18 | 0.3 | | |
| 28-2 | ~3 | 0.4 | | |
| 27-17 | ~2 | 0.5 | | |
| 56-2 | ~5 | 0.7 | | |
| 45-2 | ~4 | 1.9 | | |
| 45-8 | ~20 | 36 | | |
| 46-3 | ~16 | 39 | 0.4 | 0.3 |
| 45-1 | ~5 | 90 | 256 | 282 |
| 55-1 | ~10 | 218 | | |
| 46-2 | ~3 | 8000 | 6300 | 6750 |
| 45-5 | ~200 | 12500 | 21300 | 16700 |

The levels of hα$_1$AT in the 1st lactation milk of eleven founder transgenic mice and the second and third lactations of four of these are indicated in μg/ml.

**TABLE 2. Inheritance of AATB sequences in progeny of founder transgenic mice.**

| Line | G0 | G1 | G2 | G3 |
|---|---|---|---|---|
| 46-3 | ~16 | ~16 | ~16 (x3) | |
| 45-1 | ~5 | ~5 | ~5 (x6) | |
| | | ~5 | ~5 (x5) | |
| | | ~5 | ~5 (x9) | |
| 46-2 | ~3 | ~3 | ~3 | |
| | | ~3 | ~3 (x6) | ~3 (x2) |
| | | | | ~3 (x2) |
| 45-5 | ~200 | {~100 | {~8 | |
| | | | ~30 | |
| | | | ~20 | |
| | | | ~6} | |
| | | ~40 | ~40 | ~12 (x3) |
| | | ~100 | | |
| | | ~40 | ~20 (x3) | |
| | | ~40 | | |
| | | ~200 | | |
| | | ~5} | | |

Copy numbers of transgenes in progeny of the four founder mice analyzed are indicated. The status of progeny from any given animal is indicated in the appropriate column immediately to the right of the figure referring to the parent. Where several progeny process possess different copy numbers to their parent, figures are listed between brackets {–}. Numbers of animals with similar copy numbers is indicated by eg x2 meaning two animals.

All expressed hα$_1$AT in their milk in their first lactations although we observed an ~30,000 fold variation in human protein level. We chose to examine 4 lines with differing copy numbers and expression levels; 45-1, 45-5, 46-2 and 46-3. These were used in a breeding program to follow expression and inheritance of the transgene in sequential lactations and from generation to generation.

**Founder transgenic mice.** The G0 females which were derived from F1 male and female C57BL/6xCBA crosses were mated with F1 males (C57BL/6xCBA) and milked on day 10 post partum to derive samples from three independent lactations. Each of the chosen founder animals showed some variation of expression of hα$_1$AT between lactations (Table 1). In the milk of founders 46-2, 45-1 and 45-5, the variation in human protein was at most three-fold. Animal 45-5 was the highest copy number animal analyzed (~200) and also gave the highest yields of 12,500, 21,300 and 16,700 μg/ml of hα$_1$AT. Animal 46-2, the lowest copy number animal analyzed (~3), gave the most consistent and second highest yields of 8,000, 6,300 and 6,750 μg/ml of hα$_1$AT. Contrasting this, founder 45-1 (~5 copies of AATB) gave increasing yields of 90, 256 and 282 μg/ml and founder 46-3 (~16 copies of AATB) expressed reasonable levels of hα$_1$AT during her first lactation but subsequently failed to do so. Variations in total protein content of the milks analyzed did not account for this variation of expression (data not shown) which indicates that the levels observed reflect real differences in expression levels. Indeed, repeat assays of murine samples exhibited ≤ 10% variation (data not shown) which could not account for the observed variation in expression levels. Moreover, it is clear that the observed levels of human protein derived from AATB are not strictly copy number dependant in this system.

**Successive murine generations.** Founder females were further mated with F1 males (C57BL/6xCBA) to follow inheritance and derive milk samples from subsequent generations. Female G1 progeny were mated to F1 males (C57BL/6xCBA) to derive G2 animals and these were similarly mated to produce G3 progeny. Transgenic G1 males were mated to F1 females (C57BL/6xCBA) in order to skip a generation and the resulting transgenic female progeny mated and milked. The offspring from each of the lines were analyzed for copy number, inheritance, and expression of the transgene. The copy numbers of animals was determined by Southern blots and densitometry scanning against standards, as outlined in the Experimental Protocol, and data are summarized in Table 2. Three of the four lines appeared to inherit the transgene stably through several generations, three for 46-3 and 45-1 and four for 46-2. Only line 45.5 appeared to be unstable throughout the four generations analyzed. This was the highest copy number line examined and probably possessed multiple insertion sites as well as unstable integrants as we were unable to derive stable lines of animals by breeding this line even at the fourth generation. For this reason, expression data from this line was deemed to be of limited significance and was ignored.

**Expression of hα$_1$AT in the milk of transgenic mice.** When the expression levels of hα$_1$AT in the milk of G0 animals and their progeny were analyzed and compared, several differences were observed. As with the analysis of sequential lactations of G0 founder animals, variation in hα$_1$AT milk levels was observed in the lactations of different generations (Table 3). Samples from animals in the 46-2 line were the most consistent. Human protein levels varied by only 1.8-fold through six generations and the AATB transgene appears to be stably inherited by all animals in this line (Table 2 and unpublished results). Line 46-3 expression of AATB appeared to be shut down after the first lactation as subsequent G0 lactations yielded ~90% less hα$_1$AT

protein. The transgene appeared to be faithfully inherited through three generations and yet expression was not restored. A different situation was observed in line 45-1. The expression between generations showed a 10-fold variation despite stable inheritance of the transgene to the third generation. Female transmission of the transgene gave rise to relatively stable expression levels, ie., 90, 51 and 44 μg/ml, whereas male transmission of the transgene gave rise to a 10-fold variation. Thus, G2 female progeny derived from the G1 male of this line expressed between 54 and 520 μg/ml $h\alpha_1AT$ in their milk.

The animals used to generate the above data were all derived from F1 C57BL/6xCBA crosses. As such, genetic background may influence observed levels of expression. If this has an influence on the expression of the AATB transgene, altering the genetic background may affect the expression levels observed. To compare G1 mice with altered genetic backgrounds, the G0 mothers from lines exhibiting stable transmission of AATB were mated with CBA males. Transgenic females from their G1 litters were mated with CBA males, milked and the levels of $h\alpha_1AT$ determined (Table 4). Little effect of genetic background was observed in lines 45-1 and 46-2. In the former, the pattern of expression when the genetic background was altered reflected the variance seen at G2 in F1 x F1 crosses (~ 10-fold variation). We conclude that this line exhibits unstable expression but is unaffected by genetic background, at least in this cross. Similarly, in line 46-2, mating with a CBA male did not alter transmission of the transgene or the resulting expression level. In contrast, altering the genetic background of line 46-3 restored and increased the expression levels observed at G1 and G2 in F1 x F1 crosses. Copy numbers of all of these animals remained unaltered. Clearly, genetic background has some influence on the expression levels observed in this line. This may be the result of our use of F1 hybrids and the situation may be more consistent in similar studies using inbred lines. Genetic imprinting may also have influenced the observed variation of expression of AATB in this study[14].

These data presented above highlight some of the problems associated with the murine transgenic system as a predictive model. Our observation that genetic background may dramatically influence transgene expression levels is particularly worrying when one considers the genetic diversity of domestic livestock. If the inconsistencies seen here with murine lines 45-1 and 46-3 are to be expected when producing transgenic sheep, goats, pigs or cattle then the logistics, size and expense of an experiment designed to produce a stable, high expressing line would be truly daunting. One would hope that the situation with livestock would be more reminiscent of that seen with murine line 46-2. The following data from our sheep experiments and breeding program indicate that, at least in the case of AATB, this is indeed the case.

**AATB in transgenic sheep: inheritance of transgene and expression levels.** Transgenic sheep were generated by pronuclear injection of the AATB construct as previously described[10]. These G0 animals were derived from Blackface x East Friesland crosses. Founder females were mated with East Friesland males and after lambing were milked twice a day for the duration of their lactation period, usually about 12 weeks. They were remated to East Friesland males to derive more progeny and second lactation milk. Founders 60, 77, and 76 were also used in superovulation and embryo transfer experiments to derive further progeny from surrogate mothers. In some cases G1 females were mated to an East Friesland male to generate G2 progeny. Male founder 78 was mated to Finn Dorset ewes to generate female G1 progeny. These were mated to their transgenic G1 half-brothers to derive milk and G2 progeny, some potentially homozygous for the transgene. The relationships of animals in these various lines are summarized in Tables 5 and 6. For clarity, non-transgenic offspring are not shown but numbers of non-transgenic progeny are indicated in Tables 5 and 6.

**Transmission of AATB in ovine lines.** Blood samples were taken from all animals as a source of DNA for transgene copy number determinations which were carried out as outlined in the Experimental Protocol. Copy numbers for transgenic progeny derived from founder animals are indicated in Tables 5 and 6. We have been unable to demonstrate transmission from founder females 04 ( 15–20 copies of AATB) and 65 (< 1 copy of AATB). To date, both have produced four non-transgenic lambs. This suggests that they may both be mosaic for the transgene and may be unable to transmit to their offspring. Founder 60 has transmitted AATB to her first G1 daughter, 1LL054 . However, the copy number of the parent is 12–16 and that of the daughter is 2–4 (Table 5 and Fig. 1). This seems to have stabilized in the two sons of 1LL054 which also have 2–4 copies of AATB. Following superovulation and embryo transfer, founder 60 has also given rise to a male, 10106. He has only 8–10 copies of AATB (Table 5 and Fig. 1). This could be explained by founder 60 having two integration sites or the integrant at one site being unstable. To avoid the delay of generating more progeny, we chose to address this question by analyzing transgene integrants in animals 60,

**TABLE 3. Variation in $h\alpha_1AT$ expression in progeny of founder transgenic mice.**

| | $h\alpha_1AT$ Milk Concentration (μg/ml) | | | | | |
|---|---|---|---|---|---|---|
| Founder | G0 | G1 | G2 | G3 | G4 | G5 |
| 46-3 | 39 | 0.4 | {0.2<br>0.1} | | | |
| 45-1 | 90 | 51<br>Tg | 44<br>{54<br>316<br>520<br>72<br>116} | | | |
| 46-2 | 8000 | Tg | {6600<br>6350} | {7600<br>9200} | 5100<br>7100 | {7800<br>8100<br>7800} |
| | | | Tg | 7300}<br>8000} | | |

Concentrations of $h\alpha_1AT$ in the first lactation milk of animals are indicated in μg/ml. Figures for the progeny of any given animal are indicated in the appropriate column immediately to the right of the figure referring to the parent. Where several progeny exhibit different expression levels to their parent, they are listed between brackets {–}. Transgenic male progeny are indicated by Tg. All animals are derived from crosses to C57BL/6xCBA F1 animals. Copy numbers of all animals within a given line were equivalent.

**TABLE 4. Comparison of genetic background and $h\alpha_1AT$ protein expression.**

| Line (copy No.) | G0 Founder | G1 xC57BL/6 | G1 xCBA | G2 xC57BL/6 | G2 xCBA |
|---|---|---|---|---|---|
| 46-3 | 39 | 0.4 | | 0.2 | |
| (~16) | | | 85 | | 115<br>60 |
| 45-1 | 90 | 50 | | | |
| (~5) | | | 45<br>175<br>550 | | |
| 46-2 | 8000 | Tg | | 6600<br>6350 | |
| (~3) | | 7500 | | | |

Levels of $h\alpha_1AT$ in the first lactation milk of animals in μg/ml. Matings to F1 or CBA are indicate the column in which a figure is given. Progeny from a given animal are indicated indented to the right. Transgenic males used to pass on transgenes are indicated by Tg. Copy numbers of all animals within a given line were equivalent.

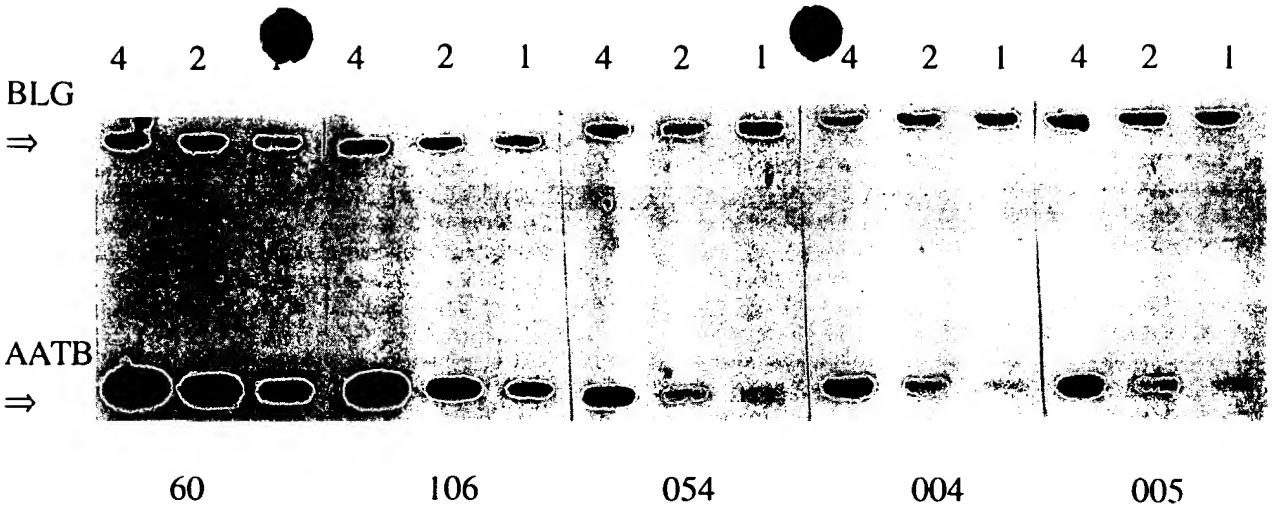


**FIGURE 1. Copy number determination for animals in the ovine AATB line 60. Three samples for each animal (numbers are indicated to the bottom of the figure) were analyzed by Southern blotting as described in the Experimental Protocol. Each lane contains a total of 4μg of ovine DNA and the number of μg of transgenic DNA in each line is indicated to the top of the figure. The band corresponding to the ovine betalactoglobulin gene is arrowed (BLG) and can be considered to represent two copies. The band representing the transgene is arrowed (AATB). Data from animal 0088 are not presented.**

1LL054 and 10106 by pulse field gel electrophoresis as described in the Experimental Protocol. If founder 60 possesses two integration events reflected in the copy numbers of 1LL054 and 10106, then this would result in two bands of different size when DNA is digested with a suitable restriction enzyme that does not cut in the injected DNA. DNA from 1LL054 should possess one of these and 10106 the other. However, all three animals have unique integrants of ~300 kbp for 60, ~175 kbp for 10106 and ~95 kbp for 1LL054 (Fig. 2). 1LL054 has given birth to two male animals, 2LL004 and 2LL005, with the same copy number as their mother. At least 2LL004 has the same sized, unique integrant of ~95 kbp as his mother (Fig. 2). Founder 60 has recently given birth to a second transgenic G1 male, 0088, having the same AATB copy number as his mother (12–16). It remains possible that the germ cells of founder 60 contain three different transgene integrants and that the DNA sample from her blood only indicates one of these. Nevertheless, this is highly unlikely and we conclude that the observed copy number differences are the result of genetic rearrangement.

The remaining founder animals 76, 77 and 78 all appear to transmit their transgene faithfully (Tables 5 and 6). Founder 76 has given rise to a G1 daughter (1LL067) and she in turn to a G2 daughter (2LL001) which both possess the same copy number as the G0 founder, 2–4. Animal 76 has also given rise to two daughters (10107, 10108) and a son (10109) by superovulation and embryo transfer. These, too, have the same copy number as their mother. Founder 77 has given rise to two sons (10110, 10111) and a daughter (10112) by superovulation and embryo transfer. Again, these have the same copy number as their mother (3–5). She has also recently given birth to a female (100) with an identical copy number to the other members of the family, 3–5. Founder male 78 initially transmitted AATB to 25% of his offspring giving rise to two transgenic males (10043, 10053) and seven transgenic females (10037, 10050, 10051, 10052, 10083, 10084 and 10085). All nine of these have 7–8

**TABLE 5. Transgenic copy number and expression levels in G0 founder female sheep and their progeny.**

| Founder G0 | G1 | G2 | Negative Progeny | Sex | Copy Number | $h\alpha_1AT$ content of milk of lactations: 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|
| 60 | | | 7 | F | 12-16 | 33.0 | 27.0 | 30.6 |
| | -10106 (SO) | | NB | M | 8-10 | | | |
| | -088 | | NB | M | 12-16 | | | |
| | -1LL054 | | 0 | F | 2-4 | 7.0 | | |
| | | -2LL004 | NB | M | 2-4 | | | |
| | | -2LL005 | NB | M | 2-4 | | | |
| 77 | | | 10 | F | 3-5 | 3.0 | 3.2 | 3.0 |
| | -10111 (SO) | | NB | M | 3-5 | | | |
| | -10112 (SO) | | NB | F | 3-5 | | | |
| | -100 | | NB | F | 3-5 | | | |
| 76 | | | 1 | F | 2-4 | 2.9 | 3.8 | NB |
| | -1LL067 | | 0 | F | 2-4 | 3.4 | NB | |
| | | -2LL001 | ND | F | 2-4 | 0.9 | | |
| | -10107 (SO) | | NB | F | 2-4 | | | |
| | -10108 (SO) | | NB | F | 2-4 | | | |
| | -10109 (SO) | | NB | F | 2-4 | | | |
| 65 | | | 4 | F | <1 | 1.5 | 1.4 | 1.4 |
| 04 | | | 4 | F | 15-20 | 1.1 | 0.7 | |

Progeny from founder G0 female animals are indicated indented to the right for each generation. NB indicates not bred, ND indicates not determined. Levels of $h\alpha_1AT$ in milk are indicated in g/l. Expression levels for G0 animals are the averages of figures from weeks 2 to 12 from first lactations, weeks 2 to at least week 16 for second lactations and weeks 2 to 8 for third lactations. All other expression levels are the average of weeks 2 to 12. The sex of animals is indicated by "F" for female and "M" for male. SO indicates that animals were derived following superovulaton of their mothers and embryo transfer.

copies of the transgene whereas founder 78 appears to possess only 3–4 copies of AATB. He is therefore a chimera in both germline and somatic tissue. Further breeding from 78 has resulted in ten more transgenic and thirty-three non-transgenic progeny. This has confirmed his transmission rate at ~25%. The nine G1 progeny were mated amongst themselves to generate ten G2 animals. Four of these appear to be homozygotes having copy numbers of 12–16 and the remaining six have the same copy number as their G1 parents, 7–8. Of these, one hemizygous female (2LL012) and one potentially homozygous female (2LL015) have recently produced G3 animals having, again, 7–8 copies of the transgene. We have also analyzed some of these progeny by pulse field gel electrophoresis as described above. Founder animal 78, his G1 daughter, 10084, and her two G2 offspring, 2LL010 and 2LL011, all appear to have the same sized transgene array of ~ 140 kbp (Fig. 2). Following our copy number determinations, we propose that 78 is mosaic and that 2LL011 is homozygous for the transgene and this seems to be supported by the relative intensities of the bands in Figure 2.

**Expression of h$\alpha_1$AT in the milk of transgenic sheep.** Milk derived from transgenic ewes was analyzed for the presence of h$\alpha_1$AT by radial immunodiffusion or ELISA as described in the Experimental Protocol. The variation between the lines in their first lactation was only ~30-fold, compared to ~30,000-fold seen in mice (Tables 5 and 6). Even limiting the murine comparison to the highest six expressers, we still observe an ~300-fold variation in human protein expression. Three founder animals have yielded very similar levels of human protein in their milk over three lactations. Founder 65 has yielded 1.5g/l h$\alpha_1$AT in her first lactation, 1.4g/l in her second lactation and 1.4g/l in her

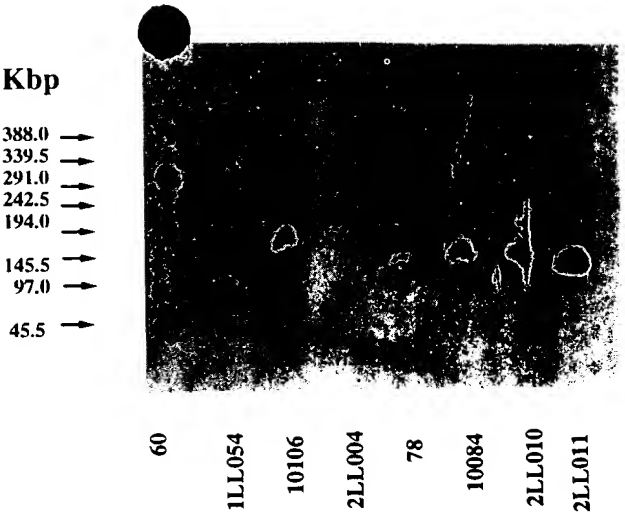


**FIGURE 2. Pulse field gel electrophoresis of DNA from animals in the ovine AATB lines 60 and 78. Samples from animals were analyzed as outlined in the Experimental Protocol and the restriction enzyme used was PacI. The resulting membrane was probed with sequences homologous to human alpha-1-antitrypsin sequences. For clarity, markers are omitted but their relative positions are indicated to the left of the diagram in Kbp. Samples presented are those from (1) G0 founder 60, two of her G1 offspring 1LL054 and 10106, and a G2 offspring derived from 21LL054, 2LL004. (2) G0 founder 78, his G1 female offspring 10084 and her two transgenic offspring 2LL010 and 2LL011. The latter is thought to be homozygous for the transgene. Note the relative band intensities in samples from 78 (mosaic), 10084 and 2LL010 (hemizygous) and 2LL011 (homozygous).**

**TABLE 6. Transgene copy number and expression levels in the G0 founder male sheep and his progeny.**

| Founder G0 | G1 | G2 | G3 | Negative Progeny | Sex | Copy Number | hα$_1$AT content of milk of lactations: 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|---|
| 78 | | | | 56 | M | 3–4 | | | |
| | -10043 | 2 | | 1 | M | 7–8 | | | |
| | -10053 | 8 | | 0 | M | 7–8 | | | |
| | -10037 | | | 0 | F | 7–8 | 16.0 | 14.0 | |
| | | -2LL006 | | NB | M | 7–8 | | | |
| | | -3LL008 | | NB | M | 7–8 | | | |
| | -10050 | | | 2 | F | 7–8 | 14.0 | 15.0 | |
| | -10051 | | | 1 | F | 7–8 | 15.0 | 17.0 | |
| | | -2LL012 | | 0 | F | 7–8 | 15.0 | | |
| | | | -81 | NB | F | 7–8 | | | |
| | | -2LL013 | | 1 | F | 7–8 | 11.8 | | |
| | | -3LL013 | | NB | F | 7–8 | | | |
| | | -3LL014 | | NB | M | 7–8 | | | |
| | -10052 | | | 1 | F | 7–8 | 15.0 | 13.0 | |
| | | -2LL014 | | NB | M | 7–8 | | | |
| | | -2LL015 | | 0 | F | 12–16 | 37.0 | | |
| | | | -87 | NB | F | 7–8 | | | |
| | -10083 | | | 2 | F | 7–8 | 14.0 | 13.0 | |
| | | -2LL008 | | NB | F | 12–16 | | | |
| | | -2LL009 | | NB | F | 12–16 | | | |
| | | -3LL003 | | NB | F | 7–8 | | | |
| | -10084 | | | 2 | F | 7–8 | 14.0 | 13.0 | |
| | | -2LL010 | | NB | M | 7–8 | | | |
| | | -2LL011 | | NB | M | 12–16 | | | |
| | -10085 | | | 1 | F | 7–8 | 13.0 | 13.0 | |
| | | -2LL021 | | NB | M | 7–8 | | | |
| | -10122 (SO) | | | NB | M | 7–8 | | | |
| | -10124 (SO) | | | NB | F | 7–8 | | | |
| | -10128 (SO) | | | NB | F | 7–8 | | | |
| | -10133 (SO) | | | NB | F | 7–8 | | | |
| | -10134 (SO) | | | NB | M | 7–8 | | | |
| | -10137 (SO) | | | NB | F | 7–8 | | | |
| | -10139 (SO) | | | NB | F | 7–8 | | | |
| | -10141 (SO) | | | NB | M | 7–8 | | | |
| | -20786 (SO) | | | NB | F | 7–8 | | | |
| | -20787 (SO) | | | NB | F | 7–8 | | | |

Progeny from the founder G0 male animal, 78, are indicated indented to the right for each generation. NB indicates that animals have not been bred from. Levels of human protein in milk are indicated in g/l. The sex of animals is indicated by "F" for female and "M" for male. SO indicates that animals were derived following superovulaton of their mothers and embryo transfer. Human protein levels in milk are the average of weeks 2–16 for G0 1st lactations. Figures for G0 second lactations and G2 1st lactations are the average of weeks 2–8.

third lactation. Founder 77 has yielded 3.0g/l, 3.2g/l and 3.0g/l over her three lactations. Founder 60 expressed an average of 33g/l of $h\alpha_1AT$ in her first lactation, 27g/l during her second lactation and so far is yielding an average of 30.6g/l in her third lactation. Her daughter, 1LL054, yielded only 7g/l in her first lactation probably reflecting the reduced copy number in this animal. Founder 76 produced similar amounts of $h\alpha_1AT$ in her two lactations, the former exhibiting 2.9g/l and the latter 3.8g/l of $h\alpha_1AT$. The daughter of founder 76, 1LL067, has also produced essentially similar levels of $h\alpha_1AT$ (3.4g/l) in her first lactation as seen in the two lactations of her mother. Unexpectedly, the G2 daughter of 1LL067, 2LL001, is currently yielding only 0.9g/l of $h\alpha_1AT$. The final founder, 04, has produced 1.1g/l of $h\alpha_1AT$ in her first lactation and is producing 0.7g/l in her second lactation.

Thus, four G0 ewes have essentially repeated their initial expression patterns. Moreover, the pattern of expression has in one case been passed from mother to G1 daughter (1LL067) although her G2 daughter (2LL001) expresses the transgene at a lower level than the previous two generations. Sheep breeds can in no way be compared to inbred mouse lines or hybrids crosses and there will be substantial genetic variation between these animals. Nevertheless, unlike our observations in mice, their expression levels are overall very similar. In most cases, the pattern of expression of $h\alpha_1AT$ during these lactations was similar to that observed in first lactations or those of an animals mother. For example, founder 77 initially secreted low levels $h\alpha_1AT$ ($\sim$ 1g/l) but increased output to the given figure mid-way through her first lactation[10]. This pattern of expression was repeated during her second and third lactations. The only exceptions to this are animals who have been affected by enzootic abortion (2LL001, 04 and 3LL013) which may have compromised the development of the mammary gland and hence milk quality. This situation can be controlled by vaccination and/or antibiotic regimes.

Male founder 78 was mated to Finn Dorset ewes to generate a mini-flock of G1 ewes all containing the same transgene integrant. These are derived from several mothers and can be considered to be half-sisters. They were mated to their half-brothers to generate G2 progeny and their milk analyzed for the presence of $h\alpha_1AT$. As an initial indication of expected expression levels, three virgin G1 females were artificially induced to lactate as described in the Experimental Protocol. Two of these failed to produce any "milk" but the third, 10085, yielded 26g/l of $h\alpha_1AT$ in her "milk" which resembled colostrum rather than milk in appearance. She has expressed approximatley half of this level (13.0g/l) in both her first and second natural lactations. The artificial induction of lactation in virgin animals, when successful, can thus serve as a useful indicator of expression level to be expected in a natural lactation. Remarkably, the six remaining G1 females all expressed 13–16g/l of the human protein in the milk of their first natural lactation and all seven ewes are currently expressing very similar levels during their second lactations. On average these seven animals produced 14.4g/l of $h\alpha_1AT$ in the milk of their first lactation and are currently producing an average of 14.0g/l $h\alpha_1AT$ in the milk of their second lactations. Again, there will be a substantial amount of genetic variation between these animals, probably as much as one would find in a normal breeding flock.

## Discussion

Although Gordon and Ruddle[15] first reported microinjection and germline transmission in 1981, regulation and control of inserted genes is still poorly understood. Expression of transgenes is influenced by regulatory elements in the chromosomal flanking regions. Al-Shawi et al.[16] have recovered an ectopically expressing transgene from mouse DNA and re-introduced it conferring correct expression demonstrating that the original aberrant expression was not due to mutations in the transgene. McKnight et al.[17] have demonstrated that chicken lysozyme matrix attachment regions (MARs) will regulate mouse whey acid protein (WAP) transgenes in a tissue specific manner independent of the site of integration in transgenic mice. However, it remains to be demonstrated that the same is true for hybrid transgenes. Grosveld et al.[17] in their study of the globin gene family have identified areas of the human genome responsible for conferring tissue-specific, position independent, copy number dependant expression. These elements, locus control regions (LCRs), can influence the behaviour of heterologous sequences. It is noteworthy that both MARs and LCRs can function across species barriers.

The importance of such elements, should they exist for the milk protein gene family, to the use of transgenic livestock for human protein production is obvious. The genomic, ovine BLG gene is expressed in a tissue specific manner at high levels ($>$ 1mg/ml) in all transgenic mice produced to date regardless of integration site (A. Carver, unpublished results, and J. Clark, pers. comm.). This occurs despite the fact that no sequence elements similar to MARs or LCRs have been identified within the BLG sequences used. Nevertheless, BLG expression seems less prone to integration effects than WAP which expresses in only 50% of transgenic mice in the absence of MARs[17]. Construction of hybrid gene combinations using the BLG promoter fused to either genomic, cDNA or mini-gene sequences invariably leads to a spectrum of expression levels as highlighted in our murine study. Indeed, despite some notable exceptions[11,18], the general inefficient expression of cDNA based constructs is well documented[19,20]. Such constructs may be more susceptible to control elements at or near the integration site. Co-injection of two genes as a gene "rescue"[21] has led to high level expression of the rescued intronless gene. Presumably, this is achieved by creating a region that insulates the hybrid gene from the effects of flanking regulatory elements or by as yet undefined direct effects on the adjacent hybrid gene and therefore allows regulated expression. However, position effects are still observed in this approach.

Here we report the erratic expression and transmission of our AATB transgene in mice clearly demonstrating the absence of MAR or LCR like sequences in the promoter region of the BLG gene or human sequences present in AATB[10]. Three of the four AATB lines analyzed faithfully transmitted their transgenes to their progeny and yet the expression levels of $h\alpha_1AT$ varied from lactation to lactation and from generation to generation (Tables 1, 2 and 3). Transgenic males who faithfully transmitted the transgene did not necessarily confer expression of the transgene (Table 3). This situation is reminiscent of other more limited studies where not all transgenic siblings expressed their transgene to the same level[21]. In our study, the genetic background of the animal also affected the level of expression (Table 4). Genetic imprinting has been reported to affect the expression of transgenes, sometimes in a parent-of-origin dependant fashion and $\sim$ 10–20% of all transgenes are expected to show signs of imprinting[14]. With this in mind, we note the different expression patterns observed following paternal or maternal transmission of transgenes in line 45-1. Only the line with the lowest copy number, 46.2, appeared stable in both transmission and expression levels in either genetic background. Transmission from a transgenic G1 male in this line also gave rise to expression levels in G2 females similar to those observed in the G0 founder animal, suggesting that the use of a male to generate a production flock of sheep is feasible.

The transgenic sheep produced do not share the same patterns of variance seen in mice. For a given founder animal, the expression levels seen during subsequent lactation seasons are

similar (Tables 5 and 6). Of [redacted] original founder animals, we have so far failed to observe transgene transmission in only two lines (lines 65 and 04). In three of the lines in which we have demonstrated transmission (lines 77, 76 and 78), the transgene has been inherited faithfully and the expression level seen in G1 females is similar to their founders (line 76). The only unstable line is that derived from the highest copy number and the highest expressing founder, 60. In the generation of her first two transgenic offspring, her transgene (12–16 copies) appears to have rearranged into two alleles, one of 2–4 copies in a G1 female, and one 8–10 copies in a G1 male. The G1 female (1LL054) has expressed $h\alpha_1AT$ at 7g/l and her two offspring (both male) appear to have inherited the transgene faithfully. The G1 male (10106) has yet to be mated. Founder 60 has recently given birth to a further transgenic male (0088) having the same copy number as his mother, suggesting that this transgene may not be consistently unstable when passed to progeny.

Although derived from a chimera, line 78 appears to be stable. The two generations of lambs derived from founder 78 have all inherited the transgene faithfully. Moreover, the seven G1 ewes all express similar levels and quality (G. Wright, unpublished) of human protein in the milk of their first and second lactations. A G2 hemizygous female (2LL013) in this line is expressing similar levels to her G1 mother and a G2 homozygous female (2LL015) is expressing nearly three times the level observed from her mother. These observations further support our proposal that flocks of transgenic livestock for the production of heterologous proteins are a feasible alternative to currently accepted technologies. Furthermore, such flocks should be derived from one founder male or several half-brothers containing the same integrant. The type of inheritance observed with line 78 is crucial in large animals where the rapid expansion of a flock or herd for production purposes has a prerequisite for one or more founder males. The consistent expression of $h\alpha_1AT$ by the line 78 G1 mini-flock is important because it demonstrates that a number of animals possessing the same transgene integrant produce similar expression levels in their milks, providing a suitable raw material from which to purify the human protein of interest.

What predictive value does the mouse model hold? When considered alone, the mouse data may not be convincing as an argument that expression of proteins in transgenic livestock would be consistent enough to result in a manageable process. Major areas of concern are the effect of genetic background on expression levels from the same transgene and the lack of MAR/LCR like sequences in constructs such as AATB. In mice one can address the former issue by using inbred lines which we predict would give more stable expression levels. In livestock, one will always be at the mercy of the random assortment of the gene pool that occurs during breeding. From the mouse data, we might have predicted highly variable expression in founder transgenic ewes and their offspring to be the norm. In practice, the reverse has been found to be true for AATB, despite the degree of genetic variation between the animals generated. Interestingly, two other cases of high expression levels of heterologous human protein in the lactating mammary gland of livestock following poor expression in mice have been reported. Velander et al.[18] recently demonstrated the expression of human protein C at levels of 1g/l in the milk of transgenic swine from a murine WAP promoter driven protein C cDNA construct that gave rise to only $\mu$g/ml levels in mice. Human tissue plasminogen activator has been expressed at up to 250$\mu$g/ml from a WAP-cDNA construct in transgenic mice[11]. An analogous construct driven from the goat $\beta$-casein promoter designed to express a longer acting variant of this protein containing a single asparagine to glutamine point mutation has given rise 2–3g/l of human protein in goat [redacted][11]. In this study, the expression levels observed in mice and goats were similar in terms of best expression levels, but the variation of expression level was much less in the goat (H. Meade, pers. comm.).

When considered together, these three cases of transgene expression in three different livestock species support the generality of our observations. Thus, poor expression levels in the mouse may not indicate poor expression levels in larger animals although the reason(s) for this remain obscure. One favorable factor in both our use of the ovine BLG gene and Meade's use of the $\beta$-casein promoter is the use of homologous species to generate transgenics. Whether this is significant remains to be seen. We conclude that the mouse, although the most convenient, is not a totally suitable model for the evaluation of gene expression of pseudo-milk proteins. However, it is certainly better than cell culture for which lines that mimic the secretory potential of the intact mammary gland are conspicuous by their absence. We think that levels of expression observed in the milk of G0 founder female mice should be considered a baseline estimate of the expression potential of a given construct which will probably be surpassed in livestock.

## Experimental Protocol

**Generation of transgenic lines.** Transgenic sheep were generated by pronuclear injection as described[10]. Transgenic mice were generated by pronuclear injection as described by Brinster et al.[14]. The mice were an F1 hybrid C57BL/6xCBA strain from our own breeding colony. AATB DNA was injected at 5–6$\mu$g/ml in 10mM Tris 0.1mM EDTA pH 7.5. The concentration of DNA was estimated using a fluorometer. The transgenic frequency for this transgene was typically ~15% of live births for mice and ~5% live births for sheep.

**Copy number determination.** DNA was extracted from tail biopsies (mice) or from peripheral ovine blood lymphocytes (prepared as previously described) by proteinase K digestion followed by phenol extraction. The DNA concentration was measured as above. Mouse copy numbers were calculated by comparison to standards probed with the BLG 800bp fragment previously described[10]. Sheep copy numbers were calculated by comparison to the homologous sheep BLG gene (2 copies per genome). Briefly DNA was digested with a restriction enzyme that generates different sized fragments for the endogenous BLG gene and the transgene, in this case BamH1. The fragments were separated through agarose and transferred to nitrocellulose. The blot was probed with a 1.8kbp fragment 5' to the cap site thus revealing a homologous BLG band and the transgene of different sizes. By dilution of the transgenic DNA with non-transgenic sheep DNA the intensity of the signals can be compared and the copy number calculated.

**Pulse field gel electrophoresis.** Pulse field gel electrophoresis was performed using a CHEF DR-II system (Bio-Rad). DNA for analysis was isolated from white blood cells which were fractionated from sheep blood as previously described[10]. Cells were washed thoroughly with phosphate buffered saline and mixed with an equal volume of molten, 1.6%, Incert agarose (FMC) to give a final concentration of approximately $2 \times 10^7$ cells per ml. The molten mixture was taken up into 1ml syringes with the ends removed and the agarose was allowed to set on ice. The agarose plugs were expelled from the syringe mould into a buffer containing 0.5M EDTA (pH 8.0) / 1% Sarkosyl / 100$\mu$g/ml proteinase K and incubated for 24–48 hours at 55°C with gentle agitation. Plugs were stored in this buffer, minus proteinase K, at 4°C Prior to PacI digestion, 10–12 slices (approximately 1–1.5mm in thickness) were cut into 5ml of 10mM Tris/0.1mM EDTA (pH 8.0) and incubated in three changes of this buffer over a period of 1–2 hours. Individual slices were placed in 200$\mu$l of a 1x solution of the appropriate restriction buffer and incubated on ice for 30 minutes. The buffer was removed and fresh buffer added containing 10–40 units of the desired restriction enzyme. The slices were incubated on ice for a further 30 minutes before being placed at the reaction temperature. Restriction digests were performed as directed by the manufacturer for 6–16 hours. Gel slices were loaded into the CHEF gel without further treatment. Rapid LE agarose (BRL) gels were employed (1% in 0.5xTBE). Running buffer was 0.5xTBE pre-chilled to 14°C. The conditions used were determined empirically and give good separation from 50kbp to about 800kbp: 198V (6V/cm), initial switch time 30s, final switch time 60s, linear ramp for 22 hours. Temperature held constant at 14°C. Gels were incubated in 0.5xTBE/1 $\mu$g/ml ethidium bromide for exactly 30 minutes. The gel was removed and exposed to a measured dose of 60mJ/cm$^2$ UV. Additional exposure to UV was minimized. DNA was capillary transferred to nylon re-enforced nitrocellulose (Schliecher & Schuell) and transgene integrants were revealed by probing with a fragment homologous to human alpha-1-antitrypsin sequences using standard protocols.

**Collection of milk samples.** Mice were milked on day 10 post partum after an injection of oxytocin[7]. Sheep were either machine milked or hand milked twice daily. Virgin animals were induced to lactate following

oestrus by the insertion of intravaginal sponges containing 50mg oestradiol and 250mg medroxy-progesterone acetate. The first was replaced after 21 days with a second sponge which was removed after a further 21 days. This resulted in the enlargement of the mammary gland and, in some cases, the production of a milk-like liquid. Such induced animals were milked by hand. Samples were frozen at -20°C prior to assaying.

**Measurement of h$\alpha_1$AT.** Determination of h$\alpha_1$AT in milk was performed as described[10]. The first week of milk in an ovine lactation contains colostrum and has an exaggerated protein concentration. Levels of human protein for sheep samples are, therefore, given as averages of results from a number of weeks of lactation as indicated in legends to Tables 5 and 6. In some cases milk is currently being analyzed and levels of human protein are averages of available results as indicated in legends to Tables 5 and 6.

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Exhibit 6

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Genetics

# High-level expression of biologically active human $\alpha_1$-antitrypsin in the milk of transgenic mice

(emphysema/elastase/antiprotease/recombinant DNA/therapeutic proteins)

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**ABSTRACT** **Reduced circulating levels of $\alpha_1$-antitrypsin ($\alpha_1$AT) are associated with certain $\alpha_1$AT genotypes and increased susceptibility to emphysema. Unfortunately, the amounts of $\alpha_1$AT that would be required for replacement therapy are beyond the capacity of plasma fractionation and mammalian cell culture systems. Thus, we have examined the potential of transgenic animals as an alternative means of producing human $\alpha_1$AT. A hybrid gene constructed by using sequences from the ovine milk protein gene $\beta$-lactoglobulin fused to an $\alpha_1$AT "minigene" was used to generate transgenic mice. Of 13 independent transgenic mice and mouse lines, 5 expressed the hybrid gene in the mammary gland, 5 in the salivary glands, and 2 in both these tissues. Human $\alpha_1$AT was secreted into the milk of each of the 7 mice and mouse lines that expressed the hybrid gene in the mammary gland. Four of these mammary-expressing transgenic mice and mouse lines produced concentrations of at least 0.5 mg of $\alpha_1$AT per ml in their milk; one line (AATB 35) produced 7 mg of this protein per ml. $\alpha_1$AT from transgenic mouse milk was similar in size to human plasma-derived $\alpha_1$AT and showed a similar capacity to inhibit trypsin. Expression at equivalent levels in transgenic sheep or cattle would yield sufficient $\alpha_1$AT for therapeutic purposes.**

Genetic deficiencies of $\alpha_1$-antitrypsin ($\alpha_1$AT) in humans are common and result in an increased susceptibility to emphysema (1). Human $\alpha_1$AT is a 394-amino acid glycoprotein that acts as a suicide inhibitor of a wide range of serine proteases. In humans, the $\alpha_1$AT gene is expressed in a variety of tissues, including macrophages, kidney, small intestine, pancreas, and liver; the latter is the primary site of expression (1, 2). In normal humans, more than 2 g of $\alpha_1$AT is synthesized daily, resulting in a serum concentration of ≈2 mg/ml.

The primary function of $\alpha_1$AT is to inhibit neutrophil elastase and thus prevent this protease from causing excessive tissue damage (1). The S and Z $\alpha_1$AT alleles are relatively common (≈0.03 and 0.02, respectively) and encode proteins that have reduced stability (S) or are poorly secreted (Z), although they exhibit normal antiprotease activity. Individuals with the SZ and ZZ genotypes have significantly reduced concentrations of $\alpha_1$AT (<0.8 mg/ml) and are at risk of developing the degenerative lung disease emphysema, particularly if they smoke.

Since $\alpha_1$AT normally circulates at 2 mg/ml and has a half-life of 6 days, considerable quantities (≈4 g per week per patient) would be required for replacement therapy for afflicted individuals (3), which amounts to 4000–8000 kg annually to treat the ZZ homozygote population of the United States (4). Such large amounts of protein will be available only if recombinant DNA technology is used for production. However, although $\alpha_1$AT does not require its carbohydrate side chains for activity, the *in vivo* half-life of nonglycosylated $\alpha_1$AT (expressed in yeast) is 50-fold lower than that of plasma-derived $\alpha_1$AT (4). Therefore it would seem prudent to produce $\alpha_1$AT in a mammalian expression system capable of making the appropriate posttranslational modifications. Unfortunately, large-scale culture of mammalian cells is expensive and technically demanding and thus far has failed to match the yields necessary for high dosage therapeutics, as exemplified by $\alpha_1$AT.

As an alternative to genetically engineered cell lines, Palmiter *et al.* (5) proposed that valuable proteins could be harvested from transgenic animals. We have argued that the mammary gland is the organ of choice for the expression of recombinant proteins (6, 7) because large amounts of protein can be synthesized by the mammary gland, secreted into milk, and collected easily without detriment to the animal. We have decided to use sheep for this purpose and have recently demonstrated the production of human factor IX and $\alpha_1$AT in the milk of transgenic sheep (8, 9). In these sheep, and also in transgenic mice carrying the same hybrid genes, the levels of expression of the transgenes were low. The comparisons of the performance of these hybrid genes (FIXA and AATA) in transgenic sheep and mice are the subject of separate studies (M.M., A.L.A., S. Harris, J.P.S., B. Whitelaw, I. Wilmut, and A.J.C., unpublished results; M.M., H. Bessos, C. Prowse, J.P.S., B. Whitelaw, I. Wilmut, and A.J.C., unpublished results).

As money and time preclude the use of large animals to test and refine DNA constructs for efficient expression, we have elected to carry out these experiments in transgenic mice. We previously showed that the gene encoding a sheep milk protein, $\beta$-lactoglobulin (BLG), was expressed efficiently and abundantly in the mammary gland of transgenic mice (10). Here we show that sequences derived from this gene can be used to direct expression of human $\alpha_1$AT sequences in the mammary gland, yielding high levels of human $\alpha_1$AT in milk.

## METHODS

**Hybrid Gene Construction and Production of Transgenic Mice.** A hybrid gene (referred to as AATB, see Fig. 1) was elaborated in which the *Pvu* II site within the 5′ untranslated sequences of the ovine BLG clone SS1 (11, 12) was fused to the *Taq* I site in the 5′ untranslated sequences of $\alpha_1$AT. The first $\alpha_1$AT intron was excluded by using DNA sequences from a cDNA clone, p8$\alpha$1ppg, which encodes the $M_1$ variant of $\alpha_1$AT (13), as the source of the first 80 base pairs of $\alpha_1$AT sequences, extending up to the *Bam*HI site in the second exon. The remainder of the $\alpha_1$AT "minigene" comprises

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Abbreviations: $\alpha_1$AT, $\alpha_1$-antitrypsin; BLG, $\beta$-lactoglobulin; G*n*, generation *n*; RID, radial immunodiffusion.

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6.5-kilobase (kb) *Bam*HI fragment from the human $\alpha_1$AT genomic clone pATp7 [also encoding the $M_1$ variant of $\alpha_1$AT (14)]. The construct was elaborated in the vector pPOLYIII-I (15), enabling excision of the 10.6-kb insert by using *Not* I sites in the polylinker sequences. Gel-purified insert DNA was microinjected into pronuclear mouse eggs [collected from (C57BL/6 × CBA)$F_1$ mice after mating with $F_1$ stud males] in order to generate transgenic mice (10, 16). Lines were propogated by mating with $F_1$ mice.

**DNA and RNA Analysis.** DNA (for Southern blot analysis) prepared from tail biopsies was digested with restriction enzyme(s), subjected to agarose gel electrophoresis, blotted to Hybond N (Amersham) nylon membranes, and probed with $^{32}$P-labeled AATB DNA sequences. RNA was prepared from lactating mice 11 days after parturition by standard methods (17, 18). Aliquots (10 $\mu$g) of total RNA were fractionated on denaturing Mops/formaldehyde (1–1.5%) agarose gels, transferred to Hybond N membranes (Amersham), and probed with a $^{32}$P-labeled 243-base-pair *Taq* I–*Pst* I fragment derived from the 3′ end of p8$\alpha$1ppg (13), which allows mouse and human $\alpha_1$AT mRNAs to be distinguished. DNA probes were labeled by using random primers (19), and hybridizations were carried out as described by Church and Gilbert (20).

**Analysis of Milk.** Milk was collected from lactating females 11 days after parturition as described by Simons *et al.* (10). Mouse milk was diluted 1:5 in distilled water, and fat was removed after centrifugation. To prepare whey, 1.0 M HCl was added to give a final pH of 4.5, to precipitate the caseins, which were then removed by centrifugation.

Diluted milk or whey samples were solubilized by boiling in loading buffer prior to discontinuous SDS/polyacrylamide (8% or 10%) gel electrophoresis (21) and immunoblotting analysis (22). Human $\alpha_1$AT was identified on immunoblot filters by using goat anti-$\alpha_1$AT serum [Protein Reference Unit (PRU), Royal Hallamshire Hospital, Sheffield S10 2JF] and anti-sheep/goat IgG serum conjugated to horseradish peroxidase [Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Lanarkshire, ML8 5ES]. Amounts of human $\alpha_1$AT in mouse milk were measured by radial immunodiffusion (RID) and radioimmunoassay (RIA). RID estimates were obtained by using LC-Partigen RID plates (Behring Diagnostics) according to the manufacturer's instructions. RIAs were performed according to standard procedures (23) with goat anti-human $\alpha_1$AT antiserum (PRU) and donkey anti-goat IgG (SAPU). Human $\alpha_1$AT, purified from plasma by using a modification of the method described by Laurell *et al.* (24), was iodinated by using chloramine T and used as the tracer, and pooled human plasma was employed for calibration. The detection limits of these methods were 40 ng/ml (RID) and 5 $\mu$g/ml (RIA), respectively, when applied to defatted murine milk samples, and results were validated by using known amounts of human plasma/serum added to control mouse milk.

**Trypsin Inhibitory Activity.** Dilutions of defatted milk or plasma (40 $\mu$l) were incubated at room temperature with equal volumes of trypsin (bovine pancreatic type III, Sigma) at 200 units/ml in 0.1 M Tris/0.15 M NaCl/3 mM sodium azide, pH 8.0 for 5 min, before addition of 40 $\mu$l of chromogenic substrate S-2222 (KabiVitrum) (25). After 2.5 min, acetic acid was added to a final concentration of 8.5% to stop the reaction, and absorbances at 405 nm were read immediately.

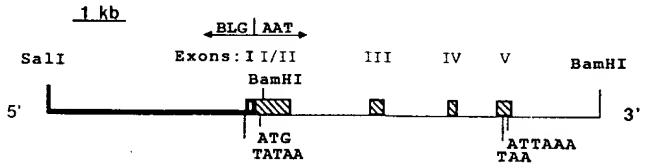


FIG. 1. The AATB construct comprises ≈4.0 kb of the 5′ end of the ovine BLG clone SS1 (11, 12) fused to a minigene encoding human $\alpha_1$AT. Thick line, 5′ BLG sequences; open box, BLG exon 1 sequences; hatched boxes, $\alpha_1$AT exons; thin lines, $\alpha_1$AT introns and 3′ flanking regions. The position of the BLG TATA box and also the $\alpha_1$AT initiation codon, stop codon, and polyadenylylation site are shown.

## RESULTS

**Generation of Transgenic Mice.** To direct expression of $\alpha_1$AT to the mammary gland of transgenic mice, a hybrid gene (AATB) was elaborated, comprising, ≈4.0 kb of the 5′ end of the ovine BLG gene fused to a minigene encoding human $\alpha_1$AT (Fig. 1). The hybrid gene (AATB) was microinjected into pronuclei of fertilized mouse eggs ($n$ = 993). Analysis of DNA prepared from tail biopsies showed that 21 of the 122 generation zero ($G_0$) animals carried the AATB construct.

**Expression of the AATB Transgene.** Expression of the transgene was assessed by analyzing RNA and milk from lactating females that were generally either $G_0$ animals or the transgenic $G_1$ offspring of $G_0$ males. Three patterns of human $\alpha_1$AT RNA expression were observed after Northern blot analysis (Fig. 2). In some animals and lines, expression was limited to the mammary gland, whereas in others it was confined to the salivary gland. There were two lines where transcripts were seen in both the mammary and the salivary glands (Table 1). As judged by comparison with human liver RNA and HepG2 RNA, both mammary and salivary transcripts were, as expected, the same size as human liver $\alpha_1$AT mRNA. One line in particular, AATB 35, showed extremely high levels of expression of $\alpha_1$AT mRNA in the mammary gland, comparable to the level observed in human liver.

**Production of Human $\alpha_1$AT in Milk.** Milk was analyzed by SDS/PAGE and immunoblotting for the presence of human $\alpha_1$AT protein (Fig. 3*a*). Human $\alpha_1$AT was present in milk from all the transgenic animals that had detectable levels of human $\alpha_1$AT mRNA in the mammary gland but was not detected in those that did not express the transgene or expressed it only in the salivary gland. The antiserum to human $\alpha_1$AT cross-reacted with an endogenous mouse protein present in milk, probably murine $\alpha_1$AT. The most

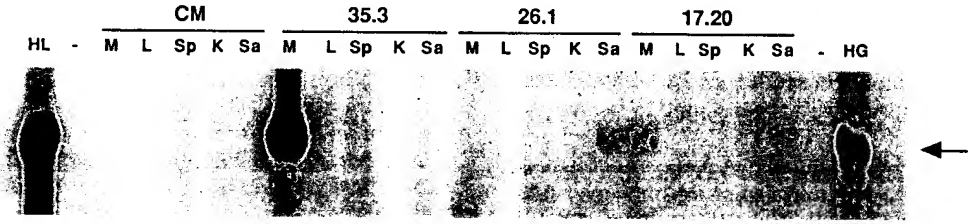


FIG. 2. Northern blot analysis of total RNA from transgenic mice (numbers AATB 35.3, AATB 26.1, and AATB 17.20) and a control C57BL/6 mouse (CM). The tissues analyzed were mammary (M), liver (L), spleen (Sp), kidney (K), and salivary (Sa). Control lanes: HL, human liver RNA; HG, HepG2 RNA (26). The ≈1400-nucleotide $\alpha_1$AT transcripts are indicated by an arrow. Ten micrograms of total RNA was loaded except HL, 35.3 M, and HG, which contain 1 $\mu$g of sample RNA with 9 $\mu$g of control mouse mammary RNA.

Table 1. Summary of the pattern of expression of AATB RNA in transgenic mice

| Animal/line | Sex* | Copy no. | Mammary | Salivary |
|---|---|---|---|---|
| 15 | M | 3 | +† | − |
| 17 | M | 10 | + | + |
| 26 | M | 20 | − | + |
| 28 | M | 3 | − | − |
| 35 | M | 1 | + | + |
| 44 | F | 15 | + | − |
| 45 | F | 2 | + | − |
| 65 | F | 3 | + | − |
| 69 | F | 2 | + | − |
| 78 | M | 10 | − | + |
| 79 | M | 15 | − | + |
| 105 | F | 20 | − | + |
| 107 | F | 15 | − | + |

Expression was analyzed by Northern blot analysis of tissues from lactating $G_0$ females or, where the founder was male, from $G_1$ females that had inherited the transgene. No human $\alpha_1$AT RNA was detected in liver, spleen, kidney, or heart. Copy numbers were estimated by Southern blotting relative to copy number controls.
*Sex of the $G_0$ animals.
†$\alpha_1$AT transcripts were detected only in poly(A)$^+$ RNA in one of two animals analyzed.

prominent human $\alpha_1$AT bands in transgenic mouse milk had electrophoretic mobilities similar, but not identical, to the major bands observed in samples of purified human $\alpha_1$AT or pooled human plasma.

The concentrations of human $\alpha_1$AT in transgenic mouse milk were measured by RID and RIA (Table 2). The results obtained with the two methods of measurement were similar. Concentrations ranged from 6 $\mu$g/ml (mouse 15.20) to more than 7 mg/ml (mouse 35.3). Of the seven animals and lines that expressed the transgene in the mammary gland, four yielded concentrations of $\alpha_1$AT of at least 0.5 mg/ml.

**Milk from Transgenic Mice Has Enhanced Trypsin-Inhibitory Activity.** Milk samples from line AATB 35 were shown to have high levels of trypsin-inhibitory activity when compared with milk from nontransgenic mice (Fig. 4*a*). When milk from line AATB 35 mice was compared with human plasma, it was evident that equivalent amounts of plasma and milk $\alpha_1$AT had similar biological activities (Fig. 4*b*). Milk from line AATB 17 mice was also shown to have greater levels of antitrypsin activity than milk from control mice. The trypsin-inhibitory capacities of milk from lines 17 and 35 were in accord with expectations based on the $\alpha_1$AT contents of these milks as measured by immunological methods.

## DISCUSSION

For the reasons outlined above, we sought to harness the high protein synthetic capacity of the mammary gland of transgenic animals as a source of recombinant $\alpha_1$AT. To this end, we elaborated a hybrid gene (AATB) by fusing the promoter and 5′ flanking sequences from the abundantly expressed ovine milk protein BLG to a human $\alpha_1$AT minigene. The construction of such hybrid genes and their excision from vectors is eased if the component sequences are kept as short as possible. However, introns have been found to be important for the expression of transgenes (ref. 27; B. Whitelaw, M.M., A.L.A., S. Harris, J.P.S., and A.J.C., unpublished results). Nevertheless, the deletion of some intron(s) may still allow high-level expression while facilitating transgene construction. The omission of the first $\alpha_1$AT (5.3-kb) intron made the elaboration of the construct simpler and excluded a 429-base-pair open reading frame, an *Alu* repeat, and a pseudo transcription initiation sequence (28).

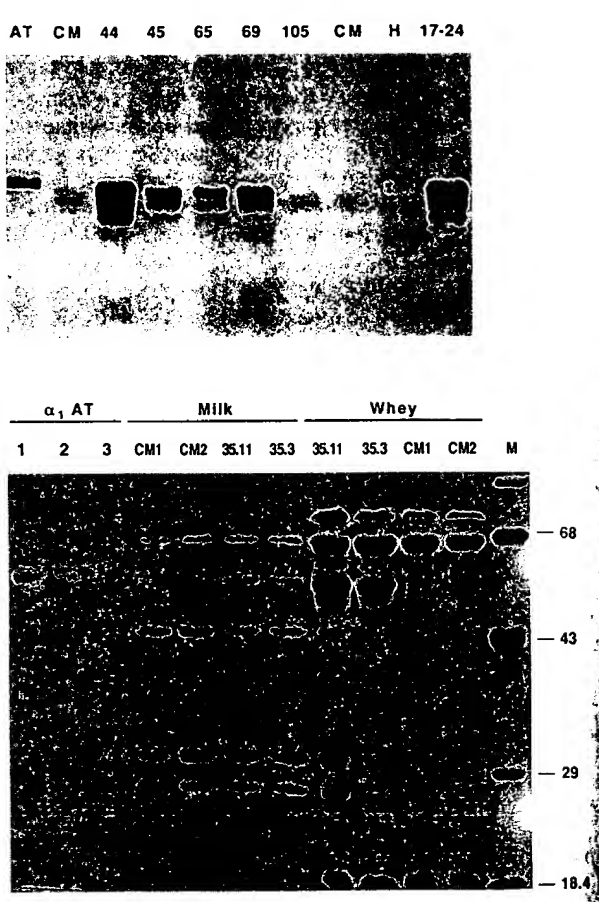


FIG. 3. Electrophoretic analysis of milk proteins. (*a*) Immunoblot. Wheys, equivalent to 1.5 $\mu$l of milk, from transgenic mice (numbered lanes) and control mice (CM), 0.25 $\mu$g of purified human $\alpha_1$AT (AT) (Sigma), and 0.05 $\mu$l of pooled human sera (H) were immunoblotted and probed for human $\alpha_1$AT. (*b*) SDS/PAGE gel. Defatted milk and whey samples from control mice (CM) and two transgenic $G_1$ females from line 35 (numbered lanes) were electrophoresed alongside dilutions of purified human $\alpha_1$AT (Sigma; lane 1, 5 $\mu$g; lane 2, 2.5 $\mu$g; lane 3, 1 $\mu$g) and molecular weight markers (M) (GIBCO, BRL) and stained with Coomassie blue.

The finding of mammary gland expression of the AATB construct in seven transgenic individuals and lines confirmed the efficacy of the construct design. However, salivary expression using the BLG promoter was not anticipated. We

Table 2. Measurements of human $\alpha_1$AT present in transgenic mouse milk as determined by immunoblotting (Blot), RID, and RIA

| Animal/line | Generation | Blot | RID, $\mu$g/ml (*n*) | RIA, $\mu$g/ml |
|---|---|---|---|---|
| 15.10 | $G_1$ | + | — (1) | 6 |
| 15.20 | $G_1$ | − | — (1) | ND |
| 17.23 | $G_1$ | + | 463 (5) | 520 |
| 17.24 | $G_1$ | + | 556 (6) | 1055 |
| 17.5.1 | $G_2$ | + | 990 (2) | 390 |
| 17.5.4 | $G_2$ | + | 407 (2) | 490 |
| 17.5.9 | $G_2$ | + | 606 (3) | 680 |
| 17.5.16 | $G_2$ | + | 730 (2) | 9000 |
| 35.3 | $G_1$ | + | 7738 (2) | 5700 |
| 35.11 | $G_1$ | + | 6215 (2) | 920 |
| 44 | $G_0$ | + | 879 (2) | 59 |
| 45 | $G_0$ | + | 84 (2) | 46 |
| 65 | $G_0$ | + | 83 (2) | 445 |
| 69 | $G_0$ | + | 695 (2) | |

*n*, Number of assays performed; ND, not determined.

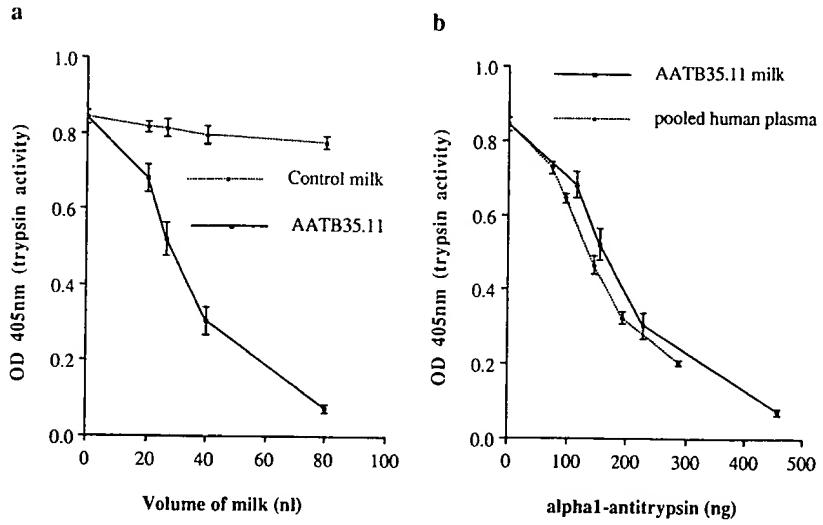


FIG. 4. Trypsin-inhibitory activity of transgenic mouse milk. (*a*) A comparison of the trypsin-inhibitory capacities of milk from transgenic mouse AATB 35.11 and pooled milk from nontransgenic mice. (*b*) A comparison of the trypsin-inhibitory capacities of the human $\alpha_1$AT in milk from mouse AATB 35.11 and in pooled human plasma; the human $\alpha_1$AT contents of the diluted milk and plasma samples were calculated from RID estimates of the concentrations of the corresponding milk and plasma.

eliminated mammary gland contamination of salivary gland RNA as the cause of this result by reprobing with mammary-specific probes (data not shown). We have not detected significant salivary expression of a variety of other transgenes comprising the BLG gene or its hybrid derivatives (ref. 10; A.J.C., A.L.A., S. Harris, M.M., J.P.S., and B. Whitelaw, unpublished observations). All these other transgenes share 3′ BLG sequences that are absent from the AATB construct. Interestingly, transgenic mice carrying the human $\alpha_1$AT gene show expression in the salivary gland (29). Several other groups have also reported salivary gland expression of hybrid genes in which the promoter and 5′ flanking sequences were also derived from mammary-specific genes (30–33). Thus, it is possible that the downstream BLG sequences contain a salivary gland-specific negative regulatory element or that positive regulatory elements within the $\alpha_1$AT sequences direct expression to this tissue.

Although the electrophoretic mobilities, in SDS/PAGE, of $\alpha_1$AT from transgenic mouse milk or human plasma are similar, the electrophoretic pattern of $\alpha_1$AT proteins observed in transgenic mouse milk appears more complex. This may reflect differences in the posttranslational modifications of the proteins produced in human liver and mouse mammary gland. Alternatively, human $\alpha_1$AT produced in mouse milk may be more susceptible to degradation during secretion or storage.

Within lines of transgenic mice, some variation in expression was observed. In line 15, low-level $\alpha_1$AT expression was detected in only one of the two animals analyzed, and in line 17, in which both $G_1$ and $G_2$ animals were analyzed, an ~2-fold variation in $\alpha_1$AT concentration was observed. This may simply reflect a variation in the total protein content of individual milk samples (10) or variation in the level of transgene expression within a line due to nonuniform genetic backgrounds. We have also noted considerable variation in the level of transgene expression within a line of mice carrying the BLG gene (M.M., unpublished observations). The level of human $\alpha_1$AT in the milk of line 35 mice is very high, as expected from the level of $\alpha_1$AT mRNA observed in the mammary gland. The $\alpha_1$AT is clearly evident on Coomassie blue-stained gels of total milk proteins (Fig. 3*b*). Densitometry of stained gels showed that human $\alpha_1$AT comprises ≈10% of total milk proteins and more than 30% of the whey proteins. These proportions compare favorably with those obtained for expression of $\alpha_1$AT in bacteria (15% of total cell protein) and yeast (3% of total soluble proteins) and particularly so when compared with eukaryotic cell culture expression (<1 μg per $10^6$ cells per 24 hr) (34–37).

The human $\alpha_1$AT present in the milk of transgenic mice from lines 17 and 35 was shown to be biologically active in a trypsin-inhibition assay. When transgenic mouse milk (mouse 35.11) and pooled human plasma were compared, it was evident that equivalent amounts of plasma and recombinant $\alpha_1$AT had similar capacities to inhibit trypsin (Fig. 4*b*), indicating that $\alpha_1$AT synthesized in the mammary gland and secreted into milk is as biologically active as that derived from plasma.

In December 1987, the U.S. Food and Drug Administration licensed the use of $\alpha_1$AT in replacement therapy (1). The methods by which $\alpha_1$AT might be delivered to the critical lung locations include intravenous infusions, aerosol sprays, and gene therapy (38, 39). It seems likely that gene therapy will be very expensive and not readily available to the many individuals suffering from $\alpha_1$AT deficiency. For replacement therapy by means of aerosols or intravenous infusions to be generally available, large quantities of biologically active and correctly glycosylated $\alpha_1$AT will be required.

In this paper we have described the production of transgenic mice expressing high levels of biologically active human $\alpha_1$AT in their milk. The levels of expression in line 35 are of particular interest and, to our knowledge, represent one of the highest levels of expression of a recombinant protein in any mammalian expression system, including transgenic mice and sheep (9, 40, 41). High-milk-yielding breeds of sheep, such as the East Friesland, can produce up to 400 liters per lactation. Therefore transgenic sheep that express $\alpha_1$AT at the levels observed in line 35 could produce up to 3 kg of $\alpha_1$AT at each lactation, a level of production that might be capable of supplying the large quantities required for replacement therapy.

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# Expression of human serum albumin in the milk of transgenic mice

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We have tested the feasibility of producing large quantities of human serum albumin (HSA) in the milk of transgenic livestock by generating transgenic mice as a model system. The sheep β-lactoglobulin (BLG) 5′-regulatory promoter sequences were used to support expression of BLG or HSA in transgenic mice. Transgenic animals generated from the entire BLG gene including 3, 5.5 or 10.8 kb of 5′-sequences demonstrated that 3 kb of 5′-sequences were sufficient to support high levels of expression of BLG, and that the longer 5′-sequences did not improve upon the levels of expression. As such, the 3 kb 5′-sequences were used to drive expression of HSA in BLG–HSA constructs. HSA was not detectably expressed in eight transgenic lines generated from a BLG–HSA construct containing the HSA cDNA. Two transgenic lines of 26 generated, using five different constructs, with an HSA minigene possessing the first intron expressed HSA in their milk. One of these expressed HSA at high levels (2.5 mg $ml^{-1}$) and has stably transmitted this ability to its progeny. A high percentage of transgenic mouse lines (four of six) generated from a vector containing an HSA minigene possessing introns 1 and 2 expressed HSA in their milk at levels which ranged from 1 to 35 μg $ml^{-1}$. In a similar trend, levels of expression of HSA by transfected tissue culture cells from BLG–HSA vectors containing an introduced SV40 enhancer were low with the HSA cDNA, increased with the HSA minigene with intron 1 and increased further with the minigene containing introns 1 and 2. This study demonstrates that high levels of HSA can be expressed in the milk of transgenic animals, that introns of the HSA gene play a role in its expression and that transfected cell lines may be used to quickly evaluate the relative expression efficiencies of various vector constructs intended for future transgenic evaluation.

Keywords: Transgenic; human serum albumin; B-lactoglobulin; milk, intron

## Introduction

The production of transgenic livestock offers a number of potential applications, one of which is 'molecular farming' (Van Brunt, 1988; also referred to as 'genetic farming') where proteins of medical or commercial importance are targeted for high level expression and production in the mammary gland with subsequent secretion into the milk of genetically engineered animals (Clark *et al.*, 1987). The feasibility of this approach was first tested in transgenic mice using the genetic regulatory elements of genes for the major milk whey proteins, sheep β-lactoglobulin (BLG) (Simons *et al.*, 1987) and mouse whey acidic protein (WAP) (Gordon *et al.*, 1987), as well as rat β-casein (Lee *et al.*, 1988). In the case of BLG and β-casein, entire genes, including regulatory and structural elements, were utilized resulting in the expression of these heterologous milk proteins in the milk of transgenic mice. On the other hand, when the WAP gene promoter region was utilized it was recombined with a human tissue plasminogen activator (t-PA) cDNA which led to the expression of active t-PA in the milk of transgenic mice. More recently, the genetic regulatory elements of other milk protein genes such as the bovine α-lactalbumin gene and the bovine $\alpha S_1$-casein gene have been used to express bovine α-lactalbumin and human urokinase, respectively, in the milk of transgenic mice (Vilotte *et al.*, 1989; Meade *et al.*, 1990). The same technology has been expanded to the expression of proteins in the milk of transgenic sheep

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(Simons *et al.*, 1988; Clark *et al.*, 1989; Wright *et al.*, 1991), rabbits (Buhler *et al.*, 1990), pigs (Wall *et al.*, 1991) and goats (Denman *et al.*, 1991; Ebert *et al.*, 1991) and is being attempted in transgenic cows (Krimpenfort *et al.*, 1991).

In most of the above cases, as well as others (Tomasetto *et al.*, 1989; Yu *et al.*, 1989; Ebert *et al.*, 1991), the levels of expression have been disappointingly low (less than 1 mg $ml^{-1}$) and in most of the instances where the non-milk protein gene was expressed at low levels, its sequences were represented as cDNAs as opposed to entire genes containing introns. In contrast, high level expression of the non-milk proteins, human $\alpha_1$-antitrypsin (Archibald *et al.*, 1990; Wright *et al.*, 1991) and human urokinase (Meade *et al.*, 1990), into the milk of transgenic animals has been reported using genes containing all or most of their native introns. These results suggest that introns may play crucial regulatory roles in the expression of the gene in which they are located within transgenic animals.

Human serum albumin (HSA) is a globular, non-glycosylated protein (MW 65000) synthesized by the liver. Circulating at levels of 42 g $l^{-1}$, it is the most abundant serum protein. It is involved in the transport of fatty acids, amino acids, bile pigments and numerous small molecules, and its presence is required for sustaining normal bloodstream osmolarity and blood pressure. Clinically, HSA is used in large quantities to replace blood volume in acute conditions such as severe burns or those associated with surgical procedures or other trauma (Peters Jr, 1975). The current medical supply of HSA, as well as other blood proteins, are produced by the fractionation of donated human blood. Commercially, HSA has a current market price which is relatively low. However, as other blood products such as coagulation factors are produced by biotechnology, as opposed to being purified from human blood, market dynamics will likely increase the relative cost of purification of HSA from blood. From a medical point of view, although commercial preparations of HSA are treated to inactivate known infectious viruses such as hepatitis and HIV, obtaining HSA from a non-human source eliminates the risk of contamination of a yet unknown human virus. As such, alternative approaches to the production of large quantities of HSA are required.

The generation of transgenic livestock expressing HSA in their milk represents an attractive approach for producing the large quantities of HSA required. We have tested the feasibility of this approach in transgenic mice. We report here the results obtained with 40 independent transgenic strains derived from the introduction of the HSA cDNA or HSA minigenes fused to sheep BLG control elements (BLG–HSA). These results demonstrate that the sheep BLG genetic regulatory elements can be used to target the expression of large quantities of HSA to the mammary gland of genetically engineered mammals with its subsequent secretion into milk.

## Materials and methods

### CONSTRUCTION OF RECOMBINANT VECTORS

The sheep BLG gene was cloned from high molecular weight liver DNA as two *Eco* RI subgenomic fragments (5′-half approximately 4.3 kb and 3′-half approximately 4.4 kb) into the *Eco* RI site of the λgt10 vector. The two fragments were subsequently removed from λgt10 and subcloned into pGEM1, joined together at their *Eco* RI sites within the transcriptional unit, using adaptor oligonucleotides which destroyed the *Eco* RI sites at the gene's 5′- and 3′-ends and which introduced *Sal* I and *Not* I sites at these positions. A unique *Sna* BI site was introduced into the *Pvu* II site within the 5′-untranslated region of BLG exon 1. This resulted in vector p585 which was used for the expression of β-lactoglobulin. The BLG 5′-flanking sequences were extended by cloning from sheep liver DNA a *Hind* III subgenomic fragment extending from the *Hind* III site within the transcriptional unit upstream approximately 8 kb to a 5′-*Hind* III site (p644 and p643) or a *Sac* I subgenomic fragment extending from the most upstream *Sac* I site within the original 5′-flanking sequences approximately 8.6 kb to a further upstream *Sac* I site (p646 and p647).

The HSA cDNA was isolated from a human liver λgt11 cDNA library. The cDNA contained the complete HSA coding sequence including the prepropeptide sequences as well as 20 base pairs of 5′-untranslated and 141 base pairs of 3′-untranslated sequences. The HSA cDNA was inserted into the *Pvu* II site within the BLG 5′-untranslated region in the same orientation as the BLG coding sequence resulting in vector p575. A HSA genomic DNA fragment including part of exon 1, intron 1, exon 2, intron 2 and part of exon 3 was produced in a polymerase chain reaction (PCR) using a 5′-oligonucleotide primer which overlaps the native *Bst* EII site in exon 1 and a 3′-oligonucleotide primer which overlaps the native *Pvu* II site in HSA exon 3 using high molecular weight DNA purified from human lymphocytes as a template. The cDNA region between the *Bst* EII site in exon 1 encoding region and the *Pvu* II site in exon 3 encoding region was replaced with the corresponding genomic PCR fragment (2401 bp). This resulted in an HSA minigene possessing introns 1 and 2 in their native positions, as included in vector p607. In order to introduce the first intron of the HSA gene into its appropriate position in the HSA cDNA, we first introduced a *Cla* I site in the region of the HSA cDNA which is derived from HSA exon 2 by replacing a G with an A in the third base position of the codon for the 34th amino acid of the HSA protein including the prepropeptide, by *in vitro* mutagenesis. The altered codon

encodes arginine as did the original. HSA intron 1 DNA with flanking exon sequences was generated by PCR using a 5'-oligonucleotide primer which overlaps the native *Bst* EII site in exon 1 and a 3'-oligonucleotide primer which overlaps and contains the *Cla* I site introduced in exon 2 encoding DNA as discussed above. A clone of the genomic PCR product containing exon 1 through exon 3 sequences discussed above was used as a template for PCR generation of intron 1 and flanking sequences. The cDNA region between the native *Bst* EII site in exon 1 encoding region and the introduced *Cla* I site in exon 2 encoding region was replaced with the corresponding genomic PCR fragment (799 bp). The resulting HSA minigene possesses intron 1 in its native position as included in vectors p599, p600, p598, p643 and p647.

The deletion of the BLG coding sequence was accomplished by deleting sequences between the introduced *Sna* BI site within the 5'-untranslated region of BLG exon 1 and the native *Xma* I site within the 3'-untranslated region of BLG exon 7 prior to introduction of HSA sequences, as seen in vectors p600 and p607. This vector maintains most of the untranslated BLG exon 7 including its polyadenylation signal as well as sequences 3' of the BLG transcription unit. The SV40 early gene polyadenylation signal downstream of HSA sequences in vectors p598, p643 and p647 was obtained from SV40 DNA by restriction with *Bcl* I at its 5'-end (SV40 map position 2770) and *Bam* HI at its 3'-end (SV40 map position 2533). In these vectors, all BLG sequences downstream of the introduced *Sna* BI site in the 5'-untranslated BLG exon 1 including coding sequence, polyadenylation signal and site and 3'-flanking sequences were deleted.

BLG–HSA vectors capable of expressing HSA *in vitro* in tissue culture cells were constructed by introducing the SV40 enhancer into the *Not* I site at the 5'-end of the BLG 5'-flanking promoter sequences. The SV40 enhancer was obtained from construct $pSV_2CAT$ (Gorman *et al.*, 1982) as a 179 bp *Fok* I (cleaves at SV40 bp position 94) to *Pvu* II (cleaves at SV40 bp position 273) fragment. The *Fok* I site was blunted with Klenow polymerase in the presence of excess dNTPs. The purified SV40 enhancer was introduced into the *Not* I site (similarly blunted) of transgenic vectors p598, p600 and p607 immediately upstream of the BLG promoter. Restriction analysis of the resultant *in vitro* tissue culture vectors p598*enh p600*enh and p607*enh, respectively, verified that only one SV40 enhancer was introduced into each construct. The SV40 enhancer was also introduced into transgenic vector p575 by replacing the p575 DNA fragment from the upstream *Pvu* I site within pGEM to the *Asp* 718 site within the BLG promoter with the homologous fragment from construct p598*enh containing the SV30 enhancer at the upstream of *Not* I site. The resultant construct was designated p575*enh. The *in vitro* tissue culture vector pMLP–HSA was constructed by introducing the HSA cDNA downstream of the adenovirus major late promoter which was immediately preceded by an SV40 enhancer at an *Eco* RV site (Hurwitz *et al.*, 1987). A DNA fragment (approximately 850 bp) consisting of the SV40 small-T splicing signals and a downstream poly (A) site (Mulligan and Berg, 1980) was positioned downstream of the HSA cDNA in this vector.

## GENERATION AND IDENTIFICATION OF TRANSGENIC ANIMALS

To purify DNA sequences for microinjection, plasmids carrying the BLG or BLG–HSA genes were restricted with *Sal* I, and appropriate fragments were electrophoretically separated on 1.5% agarose gels, electroeluted and purified on an Elutip column (Schleicher & Schuell, Keene, NH, USA). DNA was resuspended in 10 mM Tris–HCl pH 7.5 containing 0.5 mM EDTA at a concentration of 3 μg $ml^{-1}$ and microinjected into the pronuclei of FBV/N eggs (Hogan *et al.*, 1986), which were subsequently implanted into the oviducts of CDI pseudopregnant recipient mice as described (Shani, 1985, 1986). Transgenic animals were identified by tail biopsies (2 cm) taken 3 weeks after birth. Biopsies were incubated in 1 ml of 50 mM Tris–HCl pH 8.0 containing 0.5% SDS, 0.1 M EDTA and 200 μg proteinase-K overnight at 55° C. Genomic DNA was purified from the homogenates by extraction with phenol/chloroform. Approximately 10 μg of DNA from each sample was restricted with *Bam* HI, fractionated on 0.8% agarose gel and Southern transferred to Gene Screen filters (Du Pont, Boston, MA, USA). Hybridization was performed at 42° C in 50% formamide, with a probe made from the insert of plasmid p598 radiolabelled with $^{32}$P-CTP by random priming (Boehringer Mannheim, Indianapolis, IN, USA). Filters were washed with 0.2 × SSC containing 1% SDS at 60° C, and exposed to Kodak XAR-5 film at −80° C.

### *RNA analysis*

Total RNA from various tissues of transgenic lactating mice was isolated by the LiCl/urea procedure (Auffray and Rougeon, 1980). RNA (15 μg) was fractionated on MOPS/formaldehyde agarose gels and Northern transferred to Z-probe membrane (Bio-Rad, Richmond, CA, USA) and subsequently hybridized to a $^{32}$P-labelled antisense RNA probe synthesized with an RNA labelling kit (Boehringer Mannheim, Indianapolis, IN, USA), according to the supplier's protocol, and an HSA cDNA in the $pBS^-$ plasmid (Stratagene, LaJolla, CA, USA).

### *Iodolabelling of anti-HSA antibodies*

Anti-HSA monoclonal antibodies (Cedar Lane Laboratories, Hornby, Ontario, Canada) were iodinated to a specific activity of 8.0 μCi per μg by a modification of Fraker and Speck's procedure (1978). Briefly, $^{125}$I

(0.5 mCi, Amersham, Arlington Heights, IL, USA) was added to 20 μg antibody dissolved in 50 μl phosphate buffered saline (PBS) in an IODO-GEN coated tube (Pierce, Rockford, IL, USA). The reaction was terminated after 10 min by the addition of a solution containing 2.5 M potassium iodide and 1% gelatin and then passed through a Bio Rad AG-2×8 resin to remove unincorporated $^{125}I$.

*Collection and fractionation of milk*

Milk was collected from nursing transgenic mice 10–12 days after parturition. Three hours after mothers were separated from their pups they were injected intraperitonealy with 0.3 IU oxytocin (Sigma, St Louis, MO, USA). Milk was collected 10 min later by gentle massage of the mammary gland and taken up in a capillary tube (Clark *et al.*, 1987). Milk samples were diluted 1:5 in water containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and Aprotinin (Sigma) and subsequently defatted by centrifugation. To prepare whey, the caseins were first precipitated by addition of 1 M HCl to a final pH of 4.5. Whey proteins were subsequently precipitated in 10% trichloroacetic acid (TCA), washed with acetone, and solubilized in SDS polyacrylamide gel electrophoresis (PAGE) sample buffer (Laemmli, 1970).

*Milk protein analysis*

Milk proteins were analysed for the presence of either sheep BLG or HSA. For the detection of BLG, whey samples were fractionated on 15% SDS-polyacrylamide gels (Laemmli, 1970). Proteins were either stained with Coomassie Brilliant Blue or transferred onto nitrocellulose filters in a trans-blot (Bio Rad). Filters were blocked with TBS (20 mM Tris–HCl pH 7.5, 500 mM NaCl) containing 2% bovine serum albumin (BSA, Sigma) and subsequently reacted for 2 h with rabbit anti-BLG antiserum (Nordic Immunological Laboratories, Capistrano Beach, CA, USA). The complex was incubated with goat anti-rabbit IgG (BioMakor, Rehovot, Israel) and then with rabbit peroxidase anti-peroxidase (PAP, BioMakor). Peroxidase activity was revealed using diaminobenzidine as substrate. Alternatively, sheep BLG was detected using $^{125}I$-protein A following the incubation with anti-BLG antiserum. HSA was detected in milk samples fractionated on 7.5% SDS or native polyacrylamide gels. Proteins were either stained with Coomassie Brilliant Blue or transferred onto nitrocellulose filters. The filters were blocked with 3% gelatin at 37° C and then reacted overnight at room temperature with iodinated anti-HSA monoclonal antibody (Accurate Chemical and Scientific Corp, Westbury, NY, USA). After extensive washings with TBS containing 0.5% Tween, filters were exposed to Kodak XAR-5 film at −80° C.

In situ *analysis of mammary glands*

*In situ* hybridization was performed on paraffin sections of mammary glands of lactating transgenic and control mice as described (Sassoon *et al.*, 1989). HSA RNA was detected using a $^{35}S$-UTP labelled antisense RNA synthesized from HSA cDNA with T7 polymerase.

In vitro *tissue culture expression by transient transfection assay*

Mammalian COS-7 cells were plated equally into 100 mm tissue culture dishes in DMEM medium plus 10% fetal calf serum (FCS) so that they were approximately 50–75% confluent (approximately $5\times10^6$ cells). They were incubated overnight at 37° C in a $CO_2$ incubator. The next morning the medium was replaced with 5 ml of fresh medium and cells incubated for 1–2 h. They were then transfected with BLG–HSA constructs (which included the SV40 enhancer) using the calcium phosphate technique (5 Prime − 3 Prime, Inc., Boulder, CO, USA) by the supplier's protocol. The total amount of the largest construct, p575'enh (13.6 kb), transfected into cells within a plate was 25 μg. In order to transfect equal molar amounts of smaller constructs, the amounts of each of these constructs were reduced proportionally to their size differences relative to the largest construct and the total amount of DNA for each construct was brought up to 25 μg using high molecular weight (HMW) salmon sperm (ss) DNA. Following transfection of cells for 4–5 h, cells were glycerol shocked (3 ml, 2 min) and washed according to the supplier's protocol and subsequently incubated in 10 ml of DMEM medium plus 10% FCS for 3 days.

In order to detect the transient expression and secretion of HSA, transfected cells were starved for cysteine (cys) and methionine (met) by first washing and then incubating cells in cys and met deficient DMEM (containing glutamine and 5% dialysed FCS) for 1–3 h. Following removal of medium from the cells, cells were incubated for 4–5 h in 3 ml of the above medium containing 200 μCi per ml $^{35}S$-Cys and $^{35}S$-Met (Expre$^{35}S^{35}S$ $^{35}S$-Protein labelling mix; New England Nuclear, Boston, MA, USA).

After metabolic labelling, the media were harvested from dishes and centrifuged to remove any detached cells. HSA expressed and secreted into these media was detected by immunoprecipitation using rabbit anti-HSA antibodies (DAKO immunoglobulins, Denmark). Samples were first precleared with 200 μl of 50% slurry of protein A-Sepharose beads in immunoprecipitation (IPP) buffer (20 mM Tris–HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 2 μg ml$^{-1}$ aprotinin) at 4° C for 30–60 min with rocking. Following centrifugation cleared supernatants were separated from beads and treated for 3–4 h at 4° C with rabbit anti-HSA IgG prebound to protein A-Sepharose beads. Beads were washed six times with

cold IPP buffer, resuspended in 2 × SDS-PAGE Laemmli sample buffer, heated to 95° C for 5 min and run on 8% SDS-PAGE gels. Following electrophoresis, gels were fixed (10% ethanoic acid, 25% isopropanol), treated with the fluorographic reagent Amplify (Amersham, Inc.), dried onto Whatman 3MM paper and exposed to X-ray film.

## Results

### *Recombinant transgenic expression vectors*

The transgenic expression vectors used in this study were comprised of the genetic regulatory elements of the sheep BLG gene in combination with the BLG structural gene, the HSA cDNA or HSA minigenes composed of its cDNA with one or two of its native introns. The various transgenic vector constructs are shown in Fig. 1. Constructs p585, p644 and p646 were used in order to compare the levels of expression of BLG in the milk of transgenic mice from vectors with BLG 5′-flanking promoter and potential regulatory sequences ranging from approximately 3 kb to 5.5 kb to 10.8 kb, respectively. All three constructs, containing the different lengths of 5′-sequences, possess the BLG tanscriptional unit and approximately 0.8 kb of BLG 3′-flanking sequences. As is the case with all the transgenic vectors reported here, construct components used for the production of transgenic animals were separated away from bacterial plasmid (pGEM) sequences by restriction with *Sal* I and purified by gel electrophoresis.

All HSA expression vectors were constructed by introducing HSA sequences at the *Pvu* II site within the 5′-untranslated region of BLG exon 1. In the case of construct p575, the HSA cDNA under the control of the 3 kb BLG 5′-promoter sequences was followed by the rest of the BLG transcriptional unit and BLG 3′-flanking sequences. The HSA cDNA utilized did not contain a poly(A) site. As such, transcription from this construct would produce a bi-cistronic mRNA, in which the HSA sequences precede the BLG sequences and would therefore be first to be translated. Vector p599 is similar to p575 except that it contains an HSA minigene composed of the HSA cDNA and HSA intron 1 in its native position. Additional vectors were constructed with the intron 1 bearing HSA minigene.

In order to determine the effect of deleting most of the BLG transcription unit, including all of the BLG coding sequences, p600 was constructed. BLG sequences between the site of introduction of the HSA minigene within BLG exon 1 and the downstream *Xma* I site within BLG exon 7 were deleted. The BLG poly(A) site at the downstream end of BLG exon 7 as well as BLG 3′-flanking sequences were maintained. These latter sequences were replaced with an SV40 poly(A) site in vector p598, thereby deleting all BLG sequences downstream of the HSA minigene. Vectors P643 and p647 are similar to p598 in that they contain an HSA minigene with HSA intron 1 and a downstream SV40 poly(A) site. However, they possess 5.5 kb and 10.8 kb of BLG 5′-flanking promoter sequences, respectively, rather than the 3 kb of sequences found in the previously described BLG-HSA vectors. These were constructed to determine the effect of the longer BLG 5′-sequences upon the expression of the heterologous HSA gene. We constructed vector p607, comprised of the 3 kb BLG promoter, an HSA minigene with its first two introns in their native locations and the BLG poly(A) site and 3′-flanking sequences, in order to determine the effect of the addition of a second HSA intron on the expression of HSA in the milk of transgenic mice.

### *Expression of sheep BLG in the milk of transgenic mice*

Our vectors are based upon the genetic regulatory elements contained within the 5′-flanking sequences of the sheep BLG gene. Previously, successful utilization of 4 kb of BLG 5′-flanking sequences had been demonstrated (Simons *et al.*, 1987; Clark *et al.*, 1989; Archibald *et al.*, 1990). In order to demonstrate that our basic vector with a shorter length of BLG 5′-flanking sequences (3 kb) was also efficacious, without the added complication of the expression of the heterologous HSA gene, we compared vectors p585, p644 and p646 for the expression of BLG itself.

The complete sheep BLG gene contained within the *Sal* I fragment of vector p585, including approximately 3 kb of 5′-flanking sequences and 0.8 kb of 3′-flanking sequences (Fig. 1) was used to produce transgenic mice by microinjection into fertilized mouse oocytes. Seven independent transgenics were produced with integrated sequences ranging from 1 to 10 copies, as identified and determined by Southern analysis using DNA extracted from tail biopsies (data not shown). Diluted (1:5) and defatted milk collected from lactating $G_0$ or $G_1$ females from the seven transgenic lines (Nos 30, 35, 37, 38, 39, 40 and 41) were analysed for the presence of sheep BLG by immuno-dot blot using rabbit anti-bovine BLG antibodies and iodinated protein A (Fig. 2A). All seven transgenic lines expressed sheep BLG in their milk (Fig. 2A and Table 1). Expressed levels, ranging from 1.0 mg $ml^{-1}$ (line Nos 37 and 41) to 8.5 mg $ml^{-1}$ (line No. 35), were quantitated by densitometry from the intensity of the immuno-dot blot signals relative to BLG standards and sheep milk. As expected, no signal was detected with control mouse milk which does not naturally contain BLG.

In order to determine if levels of expression of sheep BLG could be increased by increasing the length of 5′-sequences flanking the BLG transcription unit, transgenic mice were produced from vectors p644 and p646 possessing approximately 5.5 kb and 10.8 kb of

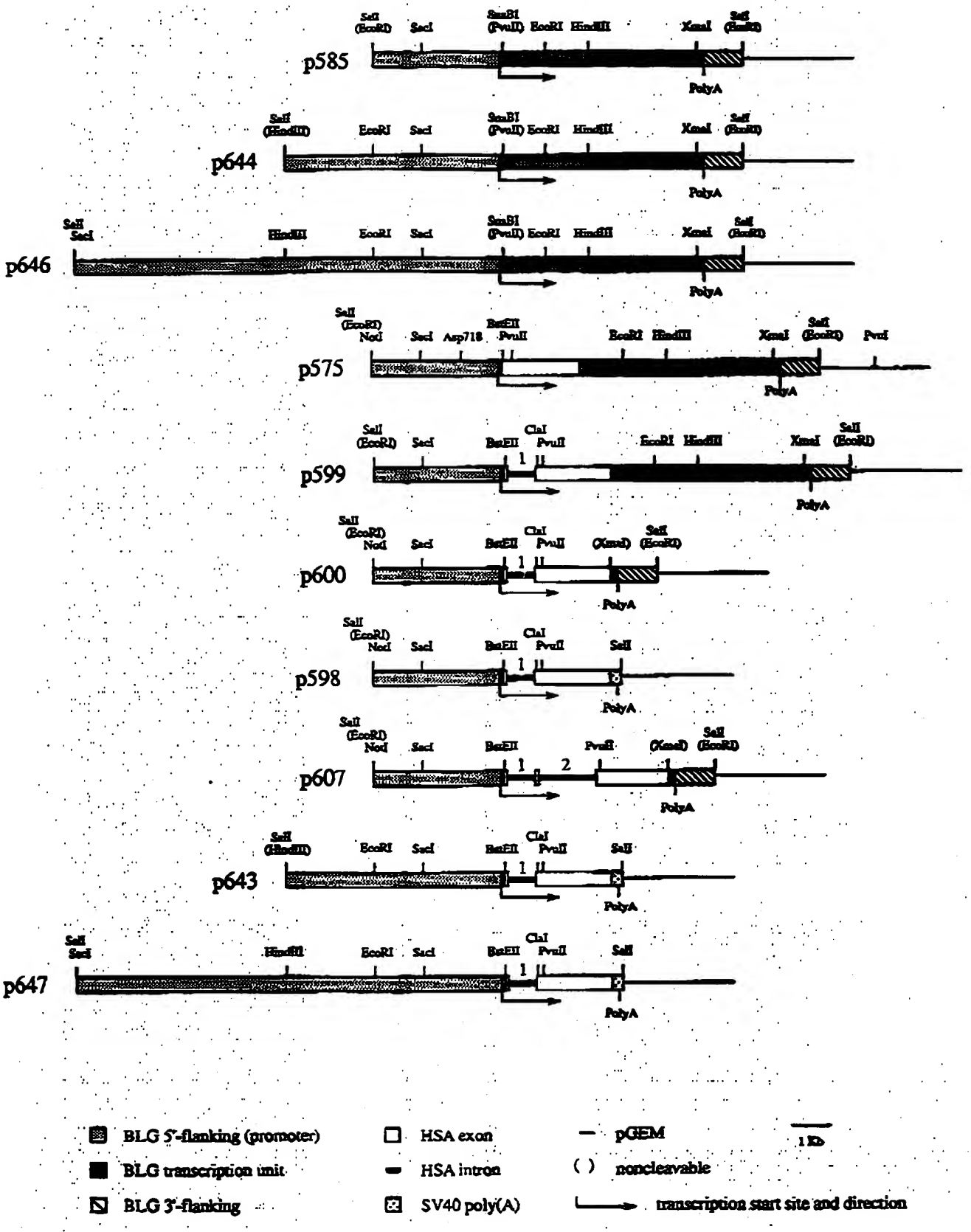


**Fig. 1.** Schematic representation of BLG–BLG and BLG–HSA expression vectors.

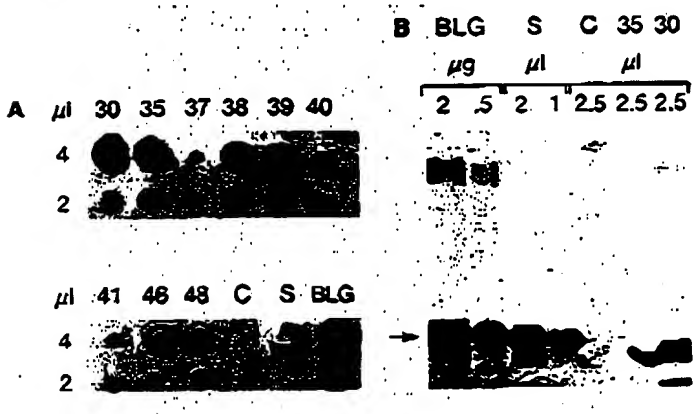


Fig. 2. Immunodetection of BLG in the milk of transgenic mice. (A) Immuno-dot blot analysis. Diluted (1:5) and defatted milk from mice and sheep was spotted onto nitrocellulose filter, dried and reacted sequentially with anti-BLG antibodies and iodinated protein-A as described in the Materials and methods section. Numbers above dots represent transgenic strain from which milk was obtained; C, control mouse milk; S, sheep milk; BLG, purified bovine BLG (Sigma, 12 μg). Numbers to the left of the dots indicate volume (μl) of milk applied. (B) SDS-PAGE and immunoblot analysis. Diluted (1:5) and defatted milk samples were decaseinated after which whey proteins were subsequently prepared as described in the Materials and methods section and subjected to SDS-PAGE (15%), blotted onto nitrocellulose filters and reacted with anti-BLG antibodies. Signal was obtained by peroxidase activity using diaminobenzidine as a substrate as described in the Materials and methods section. The arrow indicates the migration of BLG.

Table 1. Expression of BLG in the milk of transgenic mice

| *Vector* | *Transgenic strain* | *BLG* (mg ml$^{-1}$) |
|---|---|---|
| p585 | 30 | 8.4 |
| | 35 | 8.5 |
| | 37 | 1.0 |
| | 38 | 6.0 |
| | 39 | 8.3 |
| | 40 | 4.7 |
| | 41 | 1.0 |
| p644 | 43,44,49 | UD[a] |
| | 46 | 2.1 |
| | 48 | 4.2 |
| p646 | 52 | 1.0–2.0 |
| | 54 | 1.0–2.0 |
| | 56 | UD |

[a]UD, undetectable. The minimum level of detection was 2–5 μg ml.

5′-sequences, respectively. Milk samples from two of the five resultant transgenic lines produced from p644 (Nos 46 and 48) were found to contain BLG at levels within the same range as obtained from transgenics produced from vector p585 (Fig. 2A and Table 1). However, three strains from p644 (Nos 43, 44 and 49) did not express detectable BLG in their milk. In addition, two transgenic strains (Nos 52 and 54) produced from vector p646 (10.8 kb BLG 5′-sequences) expressed BLG in the range of 1–2 mg ml$^{-1}$ while a third (No. 56) did not express detectable amounts of the BLG protein (Table 1). Therefore, it appears that increasing the length of the 5′-flanking region, containing potential regulatory sequences, from 3 kb (p585) to 5.5 kb (p644) and further to 10.8 kb (p646) did not increase levels of expression of BLG.

The whey fraction of milk obtained from the two highest (Nos 30 and 35) was further analysed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with anti-BLG antiserum. An immunoreactive band of approximately 18 kDa was detected which comigrated with purified bovine BLG and the endogenous BLG in sheep milk (Fig. 2B), thus verifying the expression of authentic BLG in the milk of our transgenic mouse lines. As expected, no such band was detected in control mouse milk which does not normally contain BLG. These results indicated that our basic transgenic vector with 3 kb of 5′-flanking sequences contained sufficient information to target high level expression of protein to the mammary gland of transgenic mice, and that the 5.5 and 10.8 kb extended regions did not increase expression.

### *HSA expression in the milk of transgenic mice*

Our initial attempt to produce transgenic mice expressing HSA in their milk was by introducing the HSA cDNA into the 5′-untranslated region of the first exon of the BLG gene of vector p585, resulting in vector p575 (Fig. 1). The milk of lactating females from eight transgenic lines produced from vector p575 was analyzed for the presence of HSA by immuno-dot blot using iodinated anti-HSA monoclonal antibodies as described in the Materials and methods section. None of the eight lines secreted detectable levels of the human protein (Table 2).

It appeared that although our BLG vector was able to drive expression of its own BLG gene, it was unable to support the expression of the inserted HSA cDNA. Others have shown in transgenics that the levels of expression of heterologous genes under the control of a variety of 5′-regulatory elements (promoters) are higher when the heterologous gene contains introns than when they lack introns (Brinster *et al.*, 1988; Archibald *et al.*, 1990). As such, we decided to test a series of vectors in which the sheep BLG promoter was fused to HSA minigenes possessing either intron 1 or introns 1 and 2 within their native positions within the HSA cDNA (Fig.

Table 2. Expression of HSA in the milk of transgenic mice

| *Vector* | *Transgenic strain* | *HSA* (mg ml$^{-1}$) |
|---|---|---|
| p575 | 1–8 | UD[a] |
| p599 | 19,20,22,24,26 | UD |
| p600 | 9,11,12,14,16,17 | UD |
| p598 | 15,18,21,25 | UD |
| | 23 | 2.5 |
| p607 | 27,28 | UD |
| | 31 | 0.005 |
| | 34 | 0.001 |
| | 36 | 0.035 |
| | 42 | 0.002 |
| p643 | 45,47 | UD |
| p647 | 50,51,53,55,57,58,64 | UD |
| | 59 | 0.002 |

[a]UD, undetectable. The minimum level of detection is 0.2 μg ml$^{-1}$.

1). Vector p599 differs from vector p575 only by the presence of HSA intron 1. Vector p600 is identical to p599 except that the BLG coding sequences have been deleted whereas the untranslated BLG exon 7 with its polyadenylation signal and site as well as BLG 3′-flanking sequences have been maintained. Vector p598 is a further deletion of BLG exon 7 and 3′-flanking sequences which were replaced with an SV40 polyadenylation signal. Vectors p643 and p647 are similar to p598 except that their BLG 5′-flanking sequences extended 5.5 and 10.8 kb, respectively. Vector p607 is similar to p600 except that it includes both HSA introns 1 and 2.

From a total of 26 individual transgenic lines produced from vectors with an HSA minigene with intron 1 (p599, p600, p598, p643 and p647) only two (No. 23 from p598 and No. 59 from p647) expressed detectable levels of HSA in their milk (Table 2). The milk from line No. 23 was estimated to contain approximately 2–3 mg ml$^{-1}$ HSA as determined by comparison of its signal with HSA standards in immuno-dot blot assay and densitometry. Significantly, four of the six transgenic lines produced from vector p607, containing an HSA minigene with its first 2 introns, expressed detectable levels of HSA in their milk, ranging from 1 to 35 μg ml$^{-1}$ (Table 2).

Milk samples from five of the expressing lines, as identified by immuno-dot assay, were subjected to SDS-PAGE and immunoblot analysis (Fig. 3). An immunoreactive band comigrating with purified HSA (65 kDa) was detected in the milk of all immuno-dot positive lines and absent in control mouse milk. Densitometric analysis of the autoradiograms confirmed the quantitative estimates of HSA based upon the immuno-dot blot.

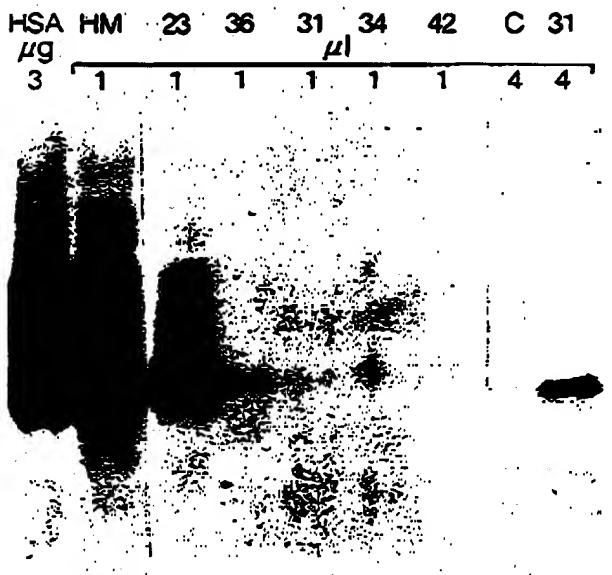


Fig. 3. SDS-PAGE (7.5%) and immunoblot analysis of HSA in the milk of transgenic mice. Samples were prepared and detected with iodinated anti-HSA antibodies as described in the Materials and methods section. HM, human milk; C, control mouse milk; HSA, purified HSA standard. Transgenic mouse strain numbers are shown at the top.

Mouse milk contains a significant amount of endogenous mouse serum albumin which comigrates with human serum albumin in SDS-PAGE gels (data not shown), however, the anti-HSA monoclonal antibody which we used for immunodetection does not react with the mouse protein (Fig. 3). The human and mouse proteins were also distinguishable by their distinct electrophoretic mobilities on native polyacrylamide gels. Milk from expressing line No. 23 clearly contains both the human (low mobility) and mouse (high mobility) albumins as seen by the staining of total milk protein samples with Coomassie Brilliant Blue (Fig. 4A). The lower mobility band was confirmed to be HSA by native gel electrophoresis followed by immunoblot analysis (Fig. 4B).

*Expression of HSA RNA in different tissues of transgenic mice*

In order to examine the tissue specificity of HSA expression, total RNA was isolated from various tissues of transgenic female mice on day 10–12 of lactation. RNAs were fractionated by electrophoresis, transferred to nylon membrane and probed with a $^{32}$P-labelled HSA antisense RNA as described in Materials and methods. The HSA probe crosshybridizes to endogenous mouse serum albumin mRNA in liver.

Two patterns of HSA RNA expression were observed as represented by trangsenic strain No. 23, produced from vector p598, whose milk contains large quantities of the HSA protein and strain No. 19, produced from vector p599, whose milk contains no detectable HSA (Fig. 5). In

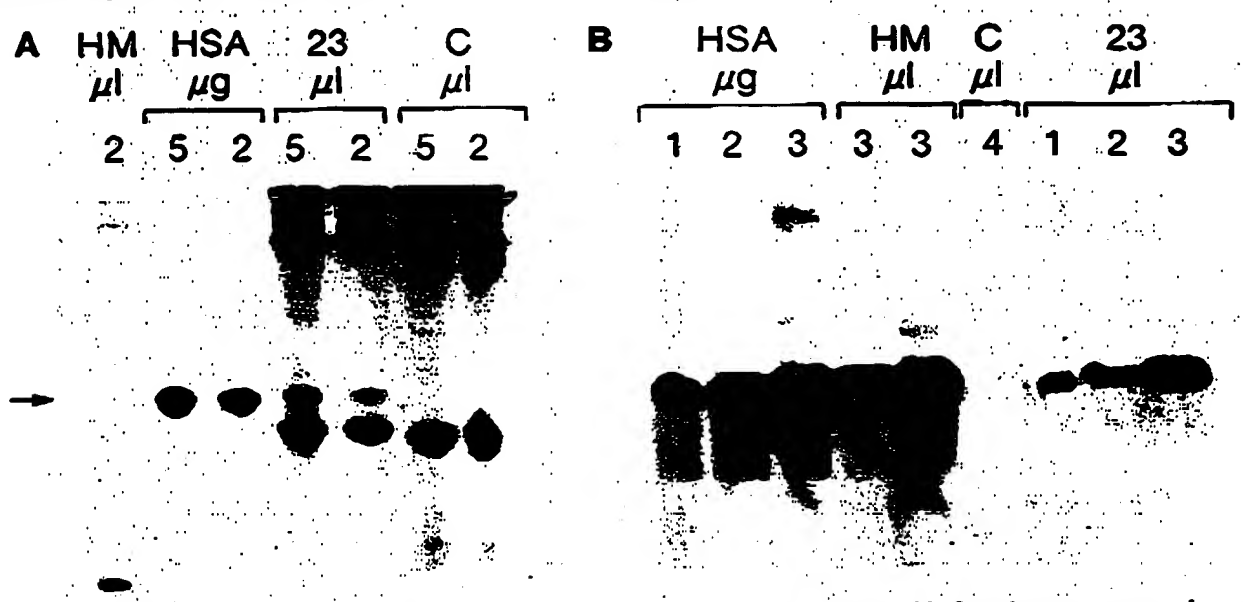


Fig. 4. Native PAGE (7.5%) analysis of HSA in the milk of transgenic mouse strain No. 23. Samples were prepared as previously described. C, control mouse milk; HM, human milk; HSA, purified HSA standard. (A) Coomassie Brilliant Blue staining of the native gel. (B) Immunoblot analysis. Proteins were blotted from gel onto nitrocellulose filters and HSA was detected with iodinated anti-HSA antibodies as described in the Materials and methods section. The arrow indicates the migration of HSA. Volumes (µl) of milk samples and amounts (µg) of HSA standards are indicated.

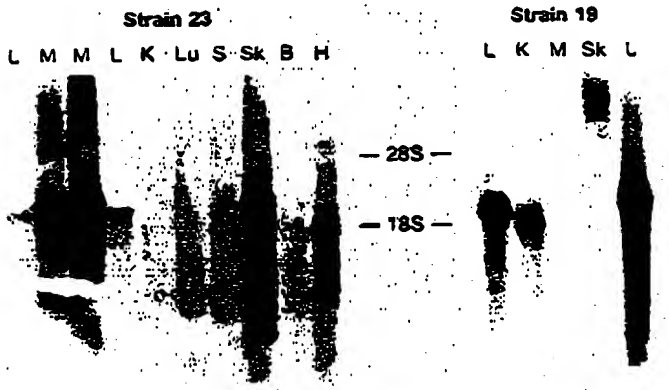


Fig. 5. Expression of HSA mRNA in various tissues of lactating transgenic mouse strain No. 23 which expresses HSA and transgenic strain No. 19 which does not express HSA. Total RNA (1 µg from liver, 15 µg from all other tissues) was fractionated by electrophoresis, transferred to nylon filters and probed with $^{32}$P-labelled HSA antisense RNA as described in the Materials and methods section. B, brain; H, heart; K, kidney; L, liver; Lu, lung; M, mammary gland; S, spleen; Sk, skeletal muscle. Relative positions of 28S and 18S ribosomal RNAs are indicated.

strain No. 23, transcripts of the transgene were clearly detected in the mammary gland and to a similar, though slightly lesser extent in skeletal muscle. No detectable signal was found in the other tissues examined, even after a long exposure of the film. Essentially the same results were found in the analysis of RNA from five additional animals of the No. 23 line. The HSA transgene RNA migrated slightly slower than the endogenous mouse serum albumin mRNA (2070 ribonucleotides). This is consistent with an expected transgenic mRNA size of about 2230 ribonucleotides composed of the untranslated portion of BLG exon 1 from its cap site to the site of introduction of the HSA minigene, the HSA transcription unit itself minus intron 1 sequences removed by splicing, and SV40 sequences upstream of the polyadenylation site.

In mouse line No. 19 (carrying vector p599), as well as six of the transgenic lines carrying vector p575 (all of whose milk contains no HSA), transgene transcripts were not detected in the mammary gland. However, significant levels of transcripts were found in the kidney. Their greater mobility than the endogenous mouse albumin in mRNA indicates an RNA smaller than the size expected (2783 ribonucleotides) of a polycistronic mRNA composed of both HSA and BLG sequences. Endogenous mouse serum albumin mRNA was also detected in the kidney of approximately 25% of control mice (data not shown).

*In situ* hybridization performed on paraffin sections of mammary glands from lactating transgenic and control mice, using an HSA specific probe, demonstrated the presence of HSA RNA in the mammary alveolar cells of transgenic strain No. 23 (Fig. 6) and its absence in the alveolar cells of control mice (data not shown). While we did not analyse the mammary development of strain No. 23 in detail, the high level of HSA secretion did not appear to affect the structure or function of the gland.

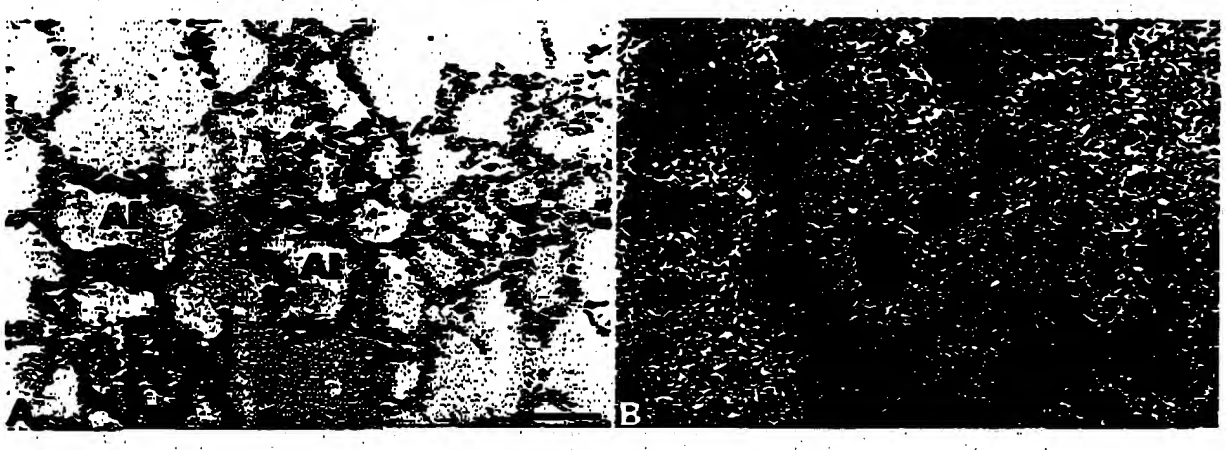


**Fig. 6.** *In situ* detection of expressed transgene RNA in the mammary gland of a lactating (days 10–12)) transgenic mouse of strain No. 23. Sections of mammary gland were probed with a $^{35}$S-UTP labelled HSA antisense RNA as described in the Materials and methods section. (A) Bright field micrograph. (B) Dark field micrograph. Al, alveoli. The bar represents 50 μm.

### In vitro *tissue culture expression of BLG–HSA vectors*

The natural *in vivo* regulation of expression of milk proteins under the control of their native promoters (e.g. BLG) is complex and requires the influence of hormones and specific cell–cell interactions. Therefore, the BLG promoter is not usually active in tissue culture cells. Thus, in order to stimulate the BLG promoter into activity, an SV40 enhancer was introduced at the *Not* I site immediately upstream of the BLG 5′-flanking sequences in transgenic vectors p575, p600, p598, and p607. The resultant tissue culture vectors were designated p575*enh, p600*enh, p598*enh and p607*enh, respectively. This allowed us to quickly test the levels of expression of the HSA protein in tissue culture cells supported by these vectors. The SV40 sequences contained within the SV40 enhancer fragment, do not include the SV40 origin of replication. As such, these vectors would not amplify within the host COS cells. An additional tissue culture vector, pMLP–HSA, was constructed to allow for the evaluation of HSA expression from its cDNA in a construct other than a BLG construct. The HSA cDNA in pMLP–HSA was placed downstream of the highly active adenovirus major late promoter and SV40 enhancer combination and upstream of the SV40 early region small-T splicing signal and poly(A) site.

The *in vitro* tissue culture expression of HSA from these vectors was evaluated using a transient expression assay following transfection of equal molar amounts of recombinant vectors into mammalian cell line COS-7. In order to detect the transient expression and secretion of HSA, cells were metabolically labelled with $^{35}$S-cysteine and $^{35}$S-methionine for 4–5 h, 3 days after transfection. HSA expressed and secreted into supernatants was immunoprecipitated with anti-HSA antibodies and evaluated by SDS-PAGE and fluorography (Fig. 7). All of the

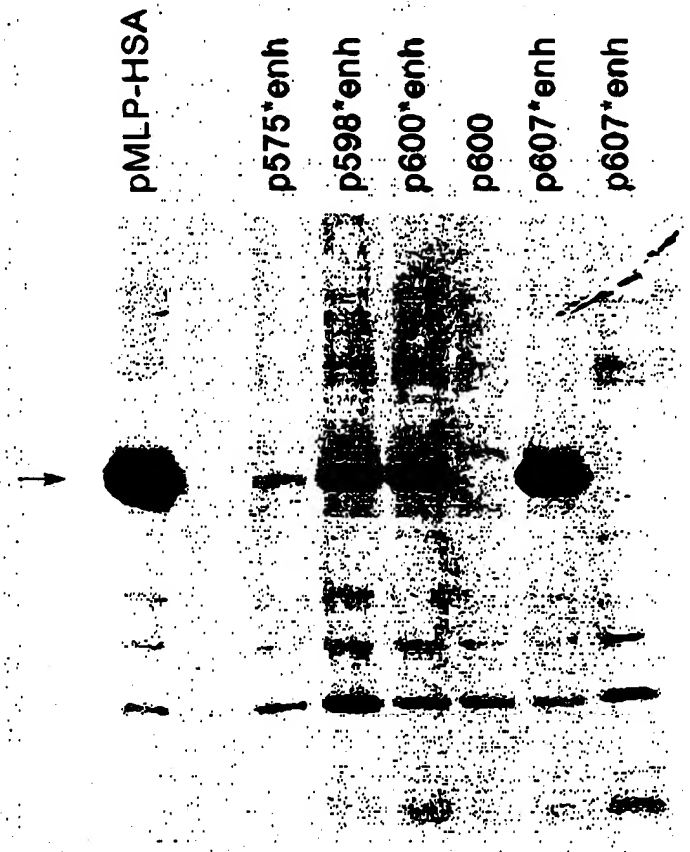


**Fig. 7.** Transient expression of HSA *in vitro* with BLG–HSA vectors. COS-7 cells were transfected with the indicated expression vectors. Expression of HSA was detected by immunoprecipitation of conditioned media from metabolically labelled cells with anti-HSA antibodies, followed by SDS-PAGE and fluorography as described in the Materials and methods section. The arrow indicates HSA band. The last lane (p607*enh) is an immunoprecipitation with an irrelevant antiserum (anti-protein tyrosine phosphatase α).

BLG–HSA tissue culture vectors supported the expression of an immunoprecipitable band (MW 65000) which co-migrated with the HSA expressed from vector pMLP-HSA. The immunoprecipitation of this band is specific to the anti-HSA antiserum and does not occur with a non-specific antiserum confirming that the band is in fact HSA. As expected, BLG–HSA transgenic vector p600, which lacks the SV40 enhancer, did not support expression of HSA in this tissue culture assay. The BLG–HSA *in vitro* vectors support a wide range of expression levels of HSA. A relatively small amount of HSA was produced from p575*enh. A dramatic increase in expression was seen with vectors p600*enh and p598*enh. The level of expression of HSA by vector p607*enh was significantly higher yet and appears to have resulted from the inclusion of a second HSA intron. These same relative expression levels were consistently obtained in several repeat experiments (data not shown).

## Discussion

In this report we describe our attempts to utilize the genetic regulatory elements of the sheep BLG gene to direct HSA synthesis to the mammary gland of transgenic mice with subsequent secretion into milk. All transgenic strains carrying the native sheep BLG gene (construct p585) with approximately 3 kb of BLG 5′-flanking sequences, expressed BLG at high levels in the mammary gland and milk. Levels ranged from 1 to 8.5 mg $ml^{-1}$ BLG. This is somewhat lower than the range (3–23 mg $ml^{-1}$) found by Simons *et al.* (1987) who utilized 4.3 kb of BLG 5′-flanking sequences. Vectors p644 and p646 were evaluated in order to determine whether extending the BLG 5′-flanking sequences beyond 3 kb to 5.5 kb and 10.8 kb, respectively, would result in higher levels of expression. An increase in BLG expression is not observed, and in fact, not all transgenics produced from p644 and p646 express BLG and those that express it do so at levels at the lower end of the range supported by p585. Possible explanations for this effect are the low sample size of transgenic lines generated from p644 and p646, the relatively large size of the vectors and/or the presence of potential inhibitory elements within these further upstream sequences. Whatever the reason(s), it appears that the incorporation of these increased lengths of BLG 5′-flanking sequences does not result in increased levels of expression. Recent work by Harris *et al.* (1990) found similar ranges of expression of BLG in the milk of transgenics carrying constructs with upstream BLG sequences of 4.3, 3.2, 2 and 0.8 kb. These results demonstrate that the BLG 3 kb 5′-flanking sequences in our constructs contain all the 5′-control elements necessary to direct high level expression to the mammary gland.

It was therefore disappointing to find that transgenics carrying a construct (p575) with the HSA cDNA introduced into the untranslated region of BLG exon 1 did not express detectable levels of HSA in their milk or HSA specific transcripts in their mammary glands. Nonetheless, these results are consistent with the observation that transgenic sheep carrying the human factor IX cDNA in the same site of the BLG gene within a similar construct, expressed only extremely low levels (25 ng $ml^{-1}$) of factor IX in their milk (Clark *et al.*, 1989). The minimum level of detection of HSA in milk by our assay is about 200 ng $ml^{-1}$. As such, it is possible that transgenic lines which we have evaluated as not having expressed HSA, do in fact express it at a level similar to, or greater than, that of factor IX (25 ng $m^{-1}$) without being detected. Low levels of expression of human proteins in the milk of transgenic mice have also resulted from constructs using the WAP promoter fused to cDNAs (Gordon *et al.*, 1987; Pittius *et al.*, 1988; Tomasetto *et al.*, 1989; Yu *et al.*, 1989). In contrast, introns have been shown to significantly increase the level of expression of mRNA from the rat growth hormone gene with two different promoter systems in transgenic mice (Brinster *et al.*, 1988). While the HSA–BLG transcriptional unit of construct p575 contains six BLG introns within the BLG sequences downstream of the HSA cDNA, the HSA cDNA itself is devoid of introns. We therefore analyzed expression from an HSA minigene, containing HSA intron 1, within 3 constructs (p599, p600, p598) which differ by their downstream sequences. Of 16 transgenic lines produced only one line (No. 23) expressed detectable levels of HSA in its milk. Apparently, HSA intron 1 alone, within an HSA minigene, is not sufficient to obtain a high percentage of transgenic lines which express HSA. Significantly, female mice of line No. 23 secrete greater than 2 mg $ml^{-1}$ of HSA into their milk and have stably transmitted this ability to their progeny for over a year. We suspect that this level of expression was due to a permissible site of integration of the transgene in the mouse genome.

The evaluation of the expression of HSA from vectors p643 and p647 comprised of an HSA minigene with intron 1 and extended BLG 5′-flanking sequences was concurrent with the evaluation of the BLG vectors p644 and p646. The fact that only 1 of 10 transgenic strains produced from p643 and p647 expressed HSA is consistent with both the lack of increased expression of BLG with the longer BLG 5′-flanking sequences and the inadequacy of HSA intron 1 alone to consistently support HSA expression.

Reproducible expression of HSA in the milk of transgenics (four of six lines) was achieved only when HSA introns 1 and 2 were included in the construct (p607). Although this is a significant improvement in the frequency of expressing transgenics, the levels of expression, in the limited number of transgenic lines evaluated, were relatively low (1–35 μg $ml^{-1}$). The fact that we did not obtain a high expressing transgenic line from p607

may simply be the result of the small number of lines generated with this vector. In other reported cases where high level expression (greater than 1 mg $ml^{-1}$) of human proteins in the milk of transgenics has been accomplished, the transgene contained all or most of its native introns (Archibald *et al.*, 1990; Meade *et al.*, 1990; Wright *et al.*, 1991). The HSA gene transcriptional unit spans approximately 17 kb and contains 14 introns (Minghetti *et al.*, 1986). The appropriate combination of these introns which will consistently direct high levels of expression is currently under analysis.

The dramatically wide range of levels of expression of HSA within the *in vitro* tissue culture assays from BLG–HSA constructs containing the SV40 enhancer was unexpected. The equal levels of expression from p600*enh and p598*enh, both with an HSA minigene containing intron 1, indicates that the BLG and SV40 3′-sequences, containing poly(A) sites, do not differentially affect expression. Levels of expression from these two vectors are strikingly higher than that supported by p575*emh containing the HSA cDNA. Vector p575*enh differs from p600*enh by the presence of the entire BLG transcriptional unit, including coding sequences, as well as the lack of an HSA intron. While it is possible that the lower level of expression of HSA from p575*enh is due to the BLG sequences, we have found an equally low level of expression of HSA from an *in vitro* vector lacking BLG sequences downstream of the HSA cDNA but including an SV40 poly(A) site (unpublished results). These results indicate that the higher level of expression of HSA from p600*enh and p598*enh over that from p575*enh is due to the presence of HSA intron 1. Vector p607*enh, with the first two HSA introns, resulted in another striking increase in expression of HSA as compared with p600*enh and p598*enh, each with only HSA intron 1. The results determined in this study suggest that the presence of HSA introns increases the level of expression of HSA within tissue culture cells and that the first two introns are more efficacious than intron 1 alone. Similarly, increased expression of the CAT gene due to the presence of an intron within the expression plasmid was seen in several tissue culture cell lines (Huang and Gorman, 1990). This increased level of expression of CAT appeared to be due to a coupling of splicing and polyadenylation pathways. The mechanism by which HSA expression in tissue culture cells is enhanced due to the presence of introns remains to be determined.

Overall, the *in vitro* tissue culture expression results and the *in vivo* transgenic expression results show similar trends. Only extremely low levels of HSA are expressed from the cDNA vector *in vitro* and no HSA is detectably expressed from the cDNA vector in transgenic mice. The inclusion of intron 1 into the *in vitro* construct results in significantly higher levels of *in vitro* expression as compared with the cDNA. Similarly, two transgenic strains derived from the transgenic construct with HSA intron 1 express HSA. One of these, No. 23, expresses HSA at high levels (2.5 mg $ml^{-1}$). The inclusion of HSA introns 1 and 2 within tissue culture constructs results in yet higher levels of expression. While these two introns have not yet resulted in particularly high levels of expression of HSA in transgenic mice, they do result in a very high percentage of transgenics which do express HSA. The similar trend between *in vitro* and *in vivo* results allows us to analyse the effects of other HSA introns upon expression of HSA in the relatively easily performed tissue culture cell assay prior to analysis in transgenic mice.

The detection of high levels of HSA mRNA in the mammary gland of lactating females which secrete HSA into their milk (line No. 23) was anticipated. HSA specific transcripts are also found in skeletal muscle but not in any of the other tissues tested (spleen, heart, kidney, lung, liver and brain). This ectopic expression of transgene transcripts is not associated with any apparent physiological abnormalities. In other cases, transgene transcripts from genes fused to the 5′-flanking sequences of the genes of milk specific proteins have been shown to accumulate in non-mammary tissues such as salivary gland, kidney and brain (Lee *et al.*, 1988; Pittius *et al.*, 1988; Vilotte *et al.*, 1989; Archibald *et al.*, 1990; Wall *et al.*, 1991). It is worth noting that we found HSA transcript accumulation in kidney tissue of several transgenics which do not express HSA in their milk. Interestingly, we also found that in one of four control mice, endogenous serum albumin mRNA was detected in kidney tissue (unpublished results). Active transcription of the endogenous albumin gene in rat kidney has been previously reported (Nahon *et al.*, 1988).

The secreted HSA protein behaves in a manner indistinguishable from purified HSA or the HSA found in human milk in its electrophoretic mobilities on native and denaturing gels. However, we found that in native gels the human protein migrates with a different mobility than the endogenous mouse serum albumin. A similar mobility difference between HSA and the serum albumin of dairy animals may simplify the purification of HSA produced in the milk of transgenic farm animals.

The level of expression of HSA in the milk of transgenic line No. 23 (2.5 mg $ml^{-1}$) is higher than the level reported for the production of HSA in *E. coli* (7% of total bacterial protein; Latta *et al.*, 1987). In addition, in *E. coli* the HSA is produced as an insoluble aggregate which requires denaturation to yield the soluble protein. No such manipulations are needed for the isolation and purification of HSA from transgenic milk. HSA has also been produced in the yeast *Saccharomyces* but at significantly lower levels and in which a high proportion of the HSA is either fragmented, cell associated or insoluble (Etcheverry *et al.*, 1986; Quirk *et al.*, 1989; Sleep *et al.*,

1990). Recently, expression of HSA in the yeast *Kluyveromyces* has reached the level of several mg per ml (Fleer *et al.*, 1991).

The expression of HSA achieved with mouse strain No. 23, as a model, is encouraging in the pursuit of the utilization of transgenic dairy farm animals as bioreactors for the efficient production of important human proteins. The similar trend between *in vitro* and transgenic expression, presented in this study, should simplify the process by which intron effects are evaluated in optimizing expression of HSA or other proteins in transgenic animals.

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